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Osteoporosis and Fracture Healing

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14. ABSTRACT This is a progress report (Year 3) for the development of a novel therapeutic drug (salubrinal and guanabenz) for skeletal diseases, focusing on potential treatment of osteoporosis and bone fracture. The study in the third year was focused on the subtasks in Tasks 3, 4, and 5, using animal models and <i>in vitro</i> cell cultures. <i>In vivo</i> data supported efficacy of salubrinal and guanabenz on strengthening bone in bone necrosis (Task 3) and bone fracture (Task 4). <i>In vitro</i> data showed genome-wide mRNA & microRNA expression patterns in response to salubrinal and guanabenz, and they indicated novel signaling molecules involved in the responses to salubrinal and guanabenz. In Task 5, a clinical trial of guanabenz is being planned at Indiana University for cancer patients with bone metastasis who suffer from severe bone loss. The study in the fourth year will be conducted focusing on Task 4 (bone fracture using local administration of the proposed agents), together with integration of <i>in vitro/in vivo</i> data for a clinical trial at Indiana University.					
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A. Main Report

Task 3: Evaluate the efficacy of salubrinal in preventing the negative effects of glucocorticoids on bone of mice compared to alendronate.

Subtask 3a. Evaluate the efficacy of salubrinal, guanabenz and alendronate (positive control) on prevention of bone loss

Comparison of salubrinal and alendronate on bone mineral density: We evaluated the effects of salubrinal using mice that received systemic administration of glucocorticoid (GC: prednisolone). Prednisolone (two dosages: GC1 – 1.4 mg/kg/day and GC2 – 2.1 mg/kg/day) and placebo pellets (Innovative Research of America) were subcutaneously implanted in the back neck region. Daily administration of salubrinal (s.c., 1 mg/kg) was conducted for 4 weeks, and alendronate (5.25 mg/kg/week) was treated as a positive control. Dual-energy x-ray absorptiometry (DXA) scans were performed five days prior to GC pellet implantation and four weeks post pellet implantation to determine changes in bone mineral density (BMD). The average percent change for each treatment condition in total body, spine, and femur BMD are shown with negative values indicating bone loss (Fig. 1).

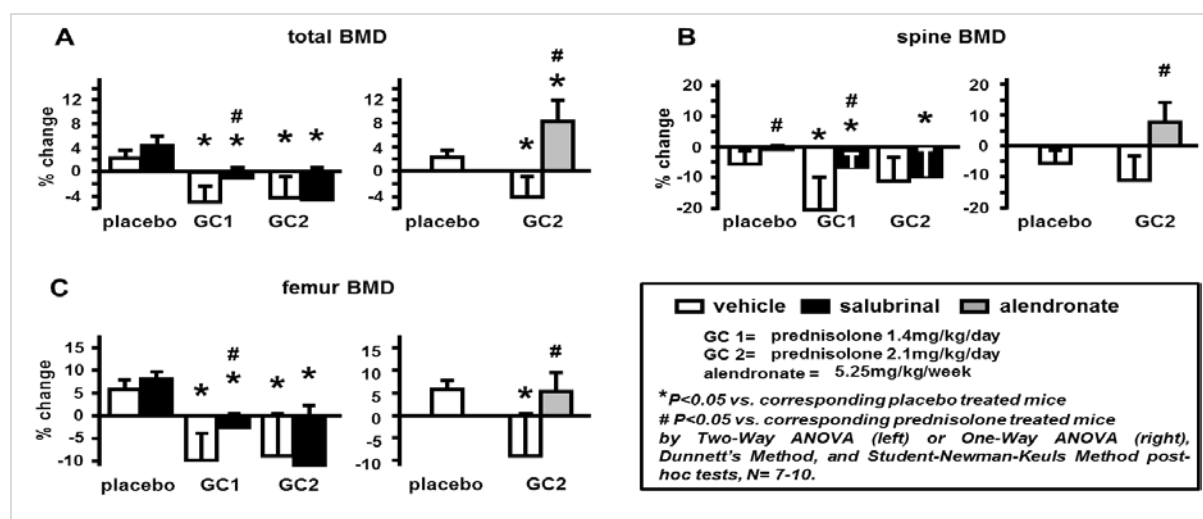
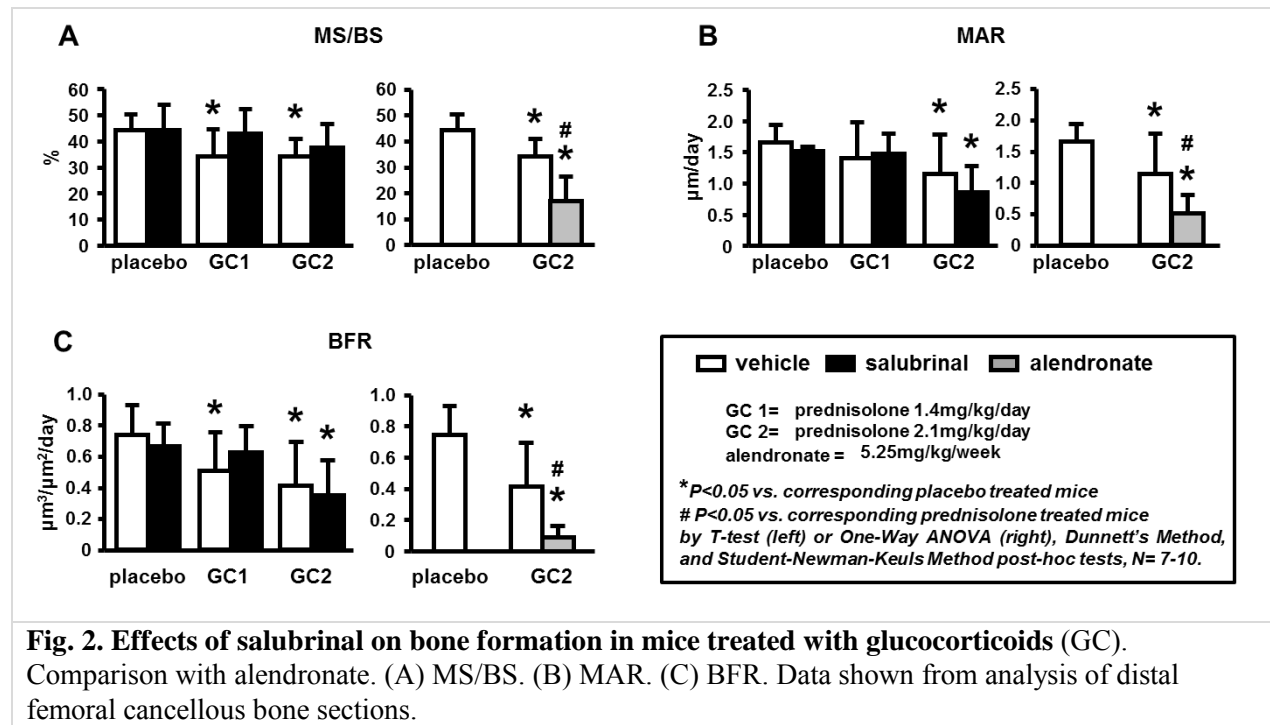


Fig. 1. Effects of salubrinal on bone mass in mice treated with glucocorticoids (GC). (A) Percent change in BMD of the total body. (B) Percent change in spinal BMD. (C) Percent change in femoral BMD.

Treatment with GC1 or GC2 significantly reduced BMD (total body, spine, and femur) except for the spine with GC2, but no clear dose response was obtained. For GC1, salubrinal suppressed GC1-driven loss of BMD in the total body, spine, and femur. For GC2, salubrinal's effect was not statistically significant. As predicted, alendronate protected against GC induced bone loss.

Comparison of salubrinal and alendronate on bone formation: We also examined salubrinal effects upon glucocorticoid-induced decreases in bone formation by dynamic histomorphometry. For this purpose, mice were injected with calcein (i.p., 0.6%) and alizarin red (i.p., 1.0%)

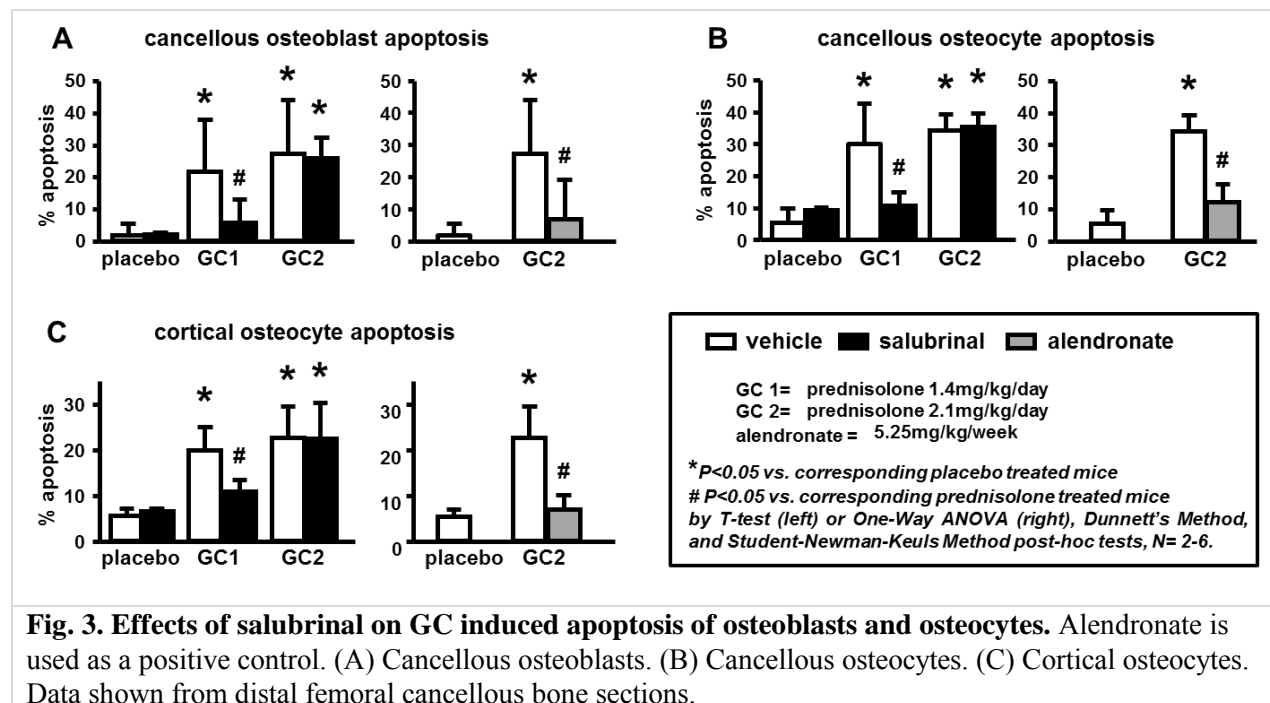
fluorochrome solutions 8 and 3 days prior to sacrifice. The mineralizing surface corrected for bone surface (MS/BS) and the mineral appositional rate (MAR) were quantified; followed by the calculation of the bone formation rate (BFR). MS/BS measures the number of osteoblasts covering the bone surfaces and MAR measures the activity of osteoblast teams. Thus, the BFR is an actual quantification of osteoblast number and activity in vivo. Results are shown in Fig. 2.



GCs administered at any of the doses used (GC1 or GC2) decreased the number of osteoblasts (MS/BS) and GC2 also decreased the activity of osteoblasts (MAR). These effects resulted in decreased BFR on cancellous bone surfaces. Salubrinal intervention protected against the effects of low dose GC1 on MS/BS and BFR resulting in a complete protection from the effects of GC1 on bone formation. However, salubrinal failed to block the decrease in MAR induced by the high dose of GC, and thus BFR was still lower in mice treated with GC2 and salubrinal. As expected based on our own published work, alendronate decreased even further BFR. These results are consistent with the suppressive effect of alendronate on bone turnover, which result in decreased bone resorption followed by decreased bone formation.

Comparison of salubrinal and alendronate on apoptosis of osteoblasts and osteocytes: We are currently determining salubrinal effects upon GC-induced apoptosis by transferase-mediated biotin-dUTP nick end-labeling (TUNEL) reaction in distal femoral bone sections. Seven-8 mice per group are being analyzed. Preliminary findings with 2-6 mice per group are shown in Fig. 3. These results show that treatment with GC markedly increased apoptosis of osteoblasts in a dose dependent manner, and that alendronate blocked the effects of GC at its higher dose (GC2). Salubrinal was able to block osteoblast apoptosis induced by GC1, but not by GC2. GC also increased apoptosis of osteocyte in cancellous and cortical bone, and again alendronate abolished

GC2 induced osteocyte apoptosis. Salubrinal effectively blocked osteocyte apoptosis induced by GC in both bone envelopes, but only at the low GC dose. In contrast, osteocyte apoptosis induced by GC2 was not affected by salubrinal.



These findings show that the decrease in bone mass and bone formation rate and the increase in osteoblast and osteocyte apoptosis induced by GC at 1.4 mg/kg/day is abolished by treatment with salubrinal. In contrast, higher doses of GC at 2.1 mg/kg/day were not able to be affected by salubrinal. Alendronate was able to block the decrease BMD and the increased osteoblast/osteocyte apoptosis induced by GC2; however, alendronate decreased even further the BFR. These results demonstrate that salubrinal and alendronate crosstalk with GC signaling by distinct mechanisms, and importantly salubrinal is able to prevent the effects of GC without decreasing the rate of bone formation.

Subtasks 3b. Investigate the molecular pathways

Genome-wide mRNA expression analysis: In order to further determine key signaling molecules in response to salubrinal/guanabenz and investigate the regulatory mechanism underlying Sal/Gu's actions, we conducted genome-wide microarray experiments focusing on mRNA expression profiles of osteoclasts (**Table 1**). In Table 1, fold-changes of mRNA expression levels are summarized among 4 groups of samples (Cont1 – control; R – RANKL treated sample; Sal – salubrinal treated sample; and Sal+R – salubrinal and RANKL treated sample). The genes in Category 1 (left column) were upregulated by RANKL (stimulator of osteoclast development), and their RANKL-driven upregulation was suppressed by Sal. Their expression levels were not significantly different in the Sal group and the control group. The genes in Category 2 (right column) were downregulated by RANKL, and their downregulation was suppressed by Sal.

Table 1. Genes whose mRNA expression levels are altered by Sal in osteoclasts.

Category 1 genes (salubrical as an inhibitor)				Category 2 genes (salubrical as a stimulator)			
Gene Symbol	Fold change			Gene Symbol	Fold change		
	Sal/Contl	R/Contl	Sal+R/Sal		Sal/Contl	R/Contl	Sal+R/Sal
Antxr2	-1.01	1.99	2.03	Snx30	1.00	-1.43	-1.43
Sdc4	-1.02	1.17	1.21	Ddit3	1.02	-1.30	-1.36
Fndc7	1.02	2.13	2.01	Litaf	-1.00	-1.38	-1.34
Zswim4	1.02	3.31	3.28	Cebpg	1.01	-1.08	-1.12
Slc25a25	1.03	1.22	1.17	Agap1	-1.01	-1.39	-1.47
Wsb2	1.02	1.39	1.39	Ppard	-1.00	-2.71	-2.63
Ado	-1.04	1.11	1.15	Ctns	1.03	-1.65	-1.75
Dusp2	1.04	1.33	1.22	Slc44a2	-1.04	-2.42	-2.44
Ifngr2	1.01	1.14	1.08	Ddit4	-1.03	-1.31	-1.15
Ppm1l	1.05	2.32	2.28	Arhgap17	1.01	-1.65	-1.61
Ipo4	1.02	1.47	1.36	Tpd52	1.04	-1.48	-1.53
Syt16	1.05	1.25	1.32	Atf3	1.04	-2.79	-3.07
Tnfrsf12a	1.11	1.36	1.25	Arl11	-1.08	-1.62	-1.52
Dhrsx	-1.04	1.13	1.11	Plekhm1	1.05	-1.44	-1.52
Tnip1	1.01	1.46	1.38	Plxdc1	-1.10	-1.16	-1.05
Pcdh7	1.09	2.50	2.37	Camk1	1.11	-1.93	-2.05
Dmwd	1.06	1.75	1.44	Tgfb1	1.20	-1.99	-2.44
Qtrt1	1.09	1.17	1.10	Pacs2	1.07	-1.43	-1.46
Jdp2	1.06	6.95	6.87	Rasa3	-1.07	-1.24	-1.24
Noc4l	1.02	1.25	1.18	Pik3r1	-1.03	-1.32	-1.34
Traf1	1.16	4.27	4.06	Pla2g15	1.10	-1.26	-1.48
Frmd6	-1.09	1.45	1.43	Xbp1	-1.02	-1.48	-1.39
Lats1	-1.06	1.15	1.14	Serp1	-1.11	-1.26	-1.18
Plaur	1.10	1.76	1.50	Hnrnpdl	-1.06	-1.07	1.04
Egr2	1.17	1.97	1.99	Tnfaip8l2	1.10	-1.77	-2.21
				Usp2	1.09	-1.56	-1.57
				Hpgds	-1.18	-3.37	-4.00

Prediction of key molecular regulators: We predicted potential Sal/Gu responsive stimulators and inhibitors of bone resorption by defining two parameters: (i) d_m for distance from NFATc1 expression pattern, and (ii) p_m for r.m.s. p-value for mRNA m . The parameter d_m indicated the resemblance ($d_m \approx 0$) and dissemblance ($d_m \approx 1$) of the mRNA expressions to the NFATc1 expression profile, and the parameter p_m indicated statistical significance. The mRNA expressions were also evaluated under 4 conditions ($j = 1$ to 4): control ($j = 1$), treatment with RANKL ($j = 2$), treatment of RANKL and salubrinal ($j = 3$), and treatment with RANKL and guanabenz ($j = 4$). All mRNAs were analyzed except for the ones with p-value > 0.05 . The distance, d_m ($0 \leq d_m \leq 1$), between NFATc1 and mRNA m , was defined using Pearson's correlation coefficient, r_m ($-1 \leq r_m \leq 1$):

$$d_m = \frac{1-r_m}{2} \quad (1)$$

$$r_m = \frac{\sum_{j=1}^n x_j y_{mj} - n \bar{x} \bar{y}_1}{\sqrt{[\sum_{j=1}^n x_j^2 - n(\bar{x})^2][\sum_{j=1}^n y_j^2 - n(\bar{y}_1)^2]}} \quad (2)$$

where x_j represented NFATc1 expression levels for $j = 1$ to n ($n = 4$), and y_{mj} was the expression levels of mRNA m for $j = 1$ to n . The variables \bar{x} and \bar{y}_1 were the mean expression levels for j . The r.m.s. p-value, p_m , for mRNA m was defined:

$$p_m = \sqrt{\frac{(p_{mR})^2 + (p_{mS})^2 + (p_{mG})^2}{3}} \quad (3)$$

where p_{mR} = p-value (Student's t-test) between $j = 1$ & 2 , p_{mS} = p-value between $j = 2$ & 3 , and p_{mG} = p-value between $j = 2$ & 4 . To better visualize the prediction result, two plots of r.m.s. p-value (p_m) vs. distance (d_m) were generated, one for the Sal/Gu-responsive osteoclastogenesis stimulators (**Fig. 4**) and one for the inhibitors (**Fig. 5**).

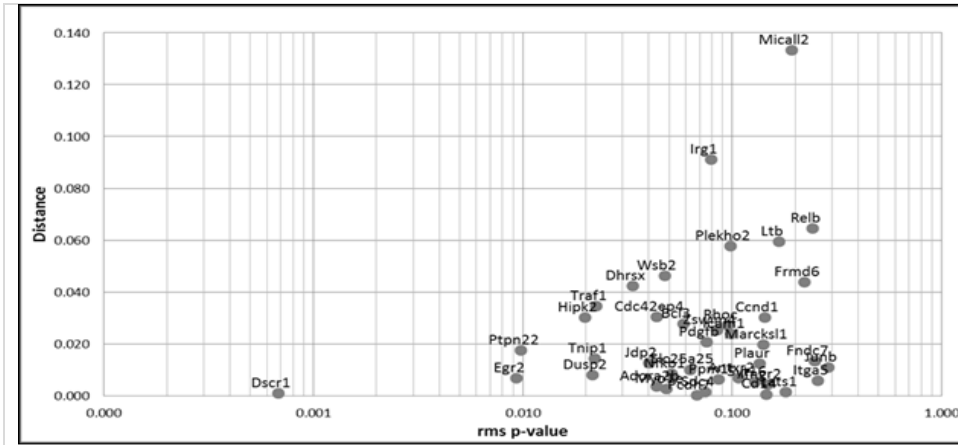


Fig. 4. Sal/Gu-responsive genes that activate RANKL-driven bone resorption. Salubrinal and guanabenz downregulate genes such as Dscr1, Dusp2, Ptpn22, etc.

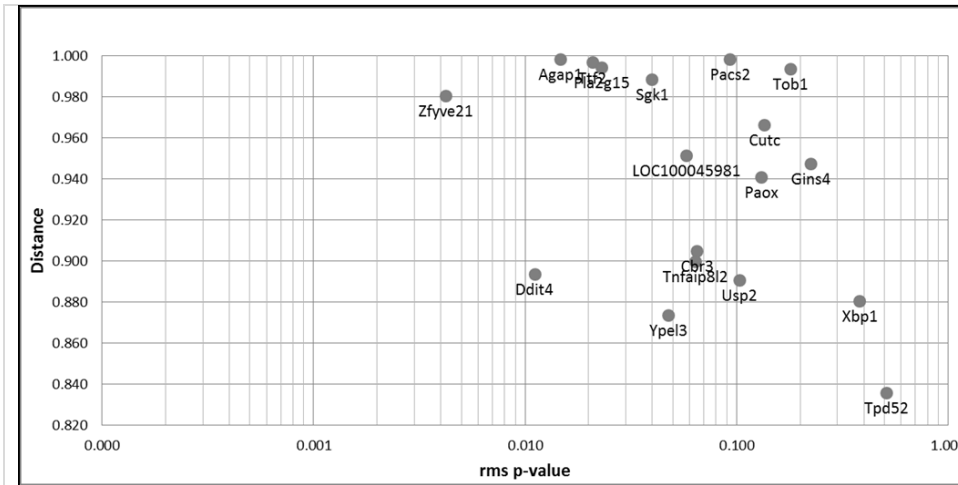
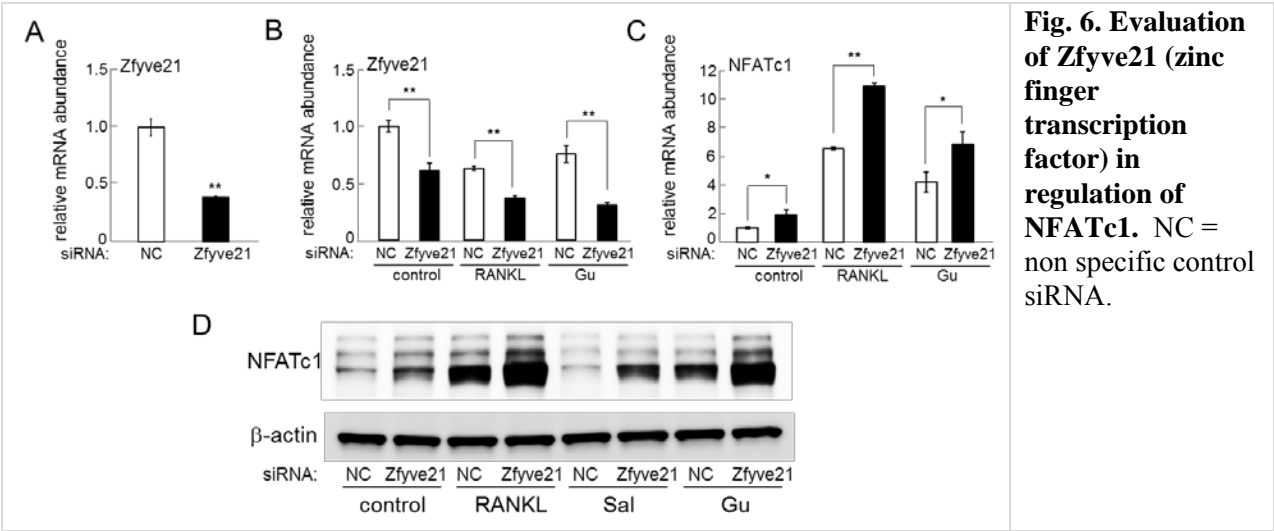
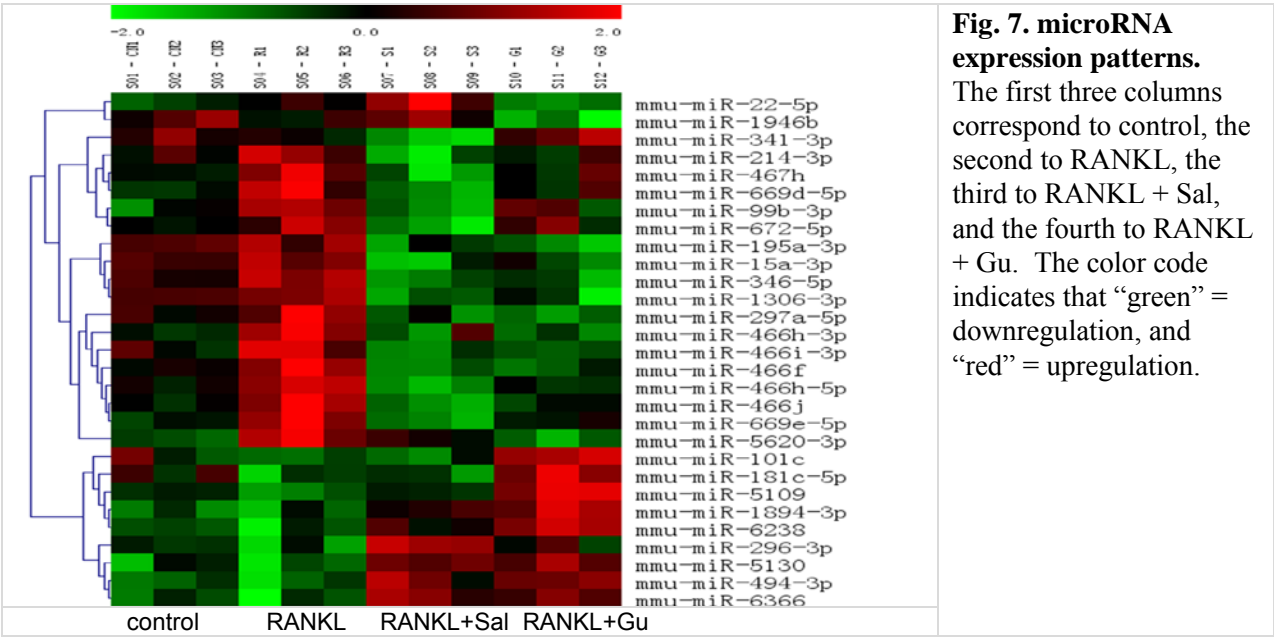


Fig. 5. Sal/Gu-responsive genes that inhibit RANKL-driven bone resorption. Salubrinal and guanabenz upregulate genes such as Zfyve21, Ddit4, etc.

Based on the predictions shown in Figs. 4 and 5, we examined the role of Zfyve21 (zinc finger transcription factor) in the regulation of NFATc1, a master transcription factor in osteoclastogenesis (**Fig. 6**). RNA interference using siRNA specific to Zfyve21 elevated the mRNA and protein levels of NFATc1, indicating that Zfyve21 at least in part inhibits NFATc1 in response to salubrinal and guanabenz.



Genome-wide microRNA analysis: Besides mRNA expression profiles, we also examined microRNA expression profiles in response to salubrinal and guanabenz. One-thousand-two-hundred-sixty-five microRNAs were inputted, and cluster analysis was conducted to characterize expression patterns among 4 groups of samples (control, RANKL, RANKL + Sal, and RANKL + Gu).



Similar to the mRNAs, the miRNAs were correlated to NFATc1 and divided into stimulator and inhibitor groups. Again, distance values of “0” indicated similarity with the NFATc1 expression pattern, and distance values of “1” indicated expression patterns opposite to NFATc1. Many candidates with significant r.m.s. p-values are shown in the NFATc1-like group, but only one, miR-5109, is found with significant r.m.s. p-value in the NFATc1-reciprocal group. Regardless of the significance of the r.m.s. p-values, miRNAs with small distance values (below 0.3 or above 0.7) were analyzed with the target prediction tool.

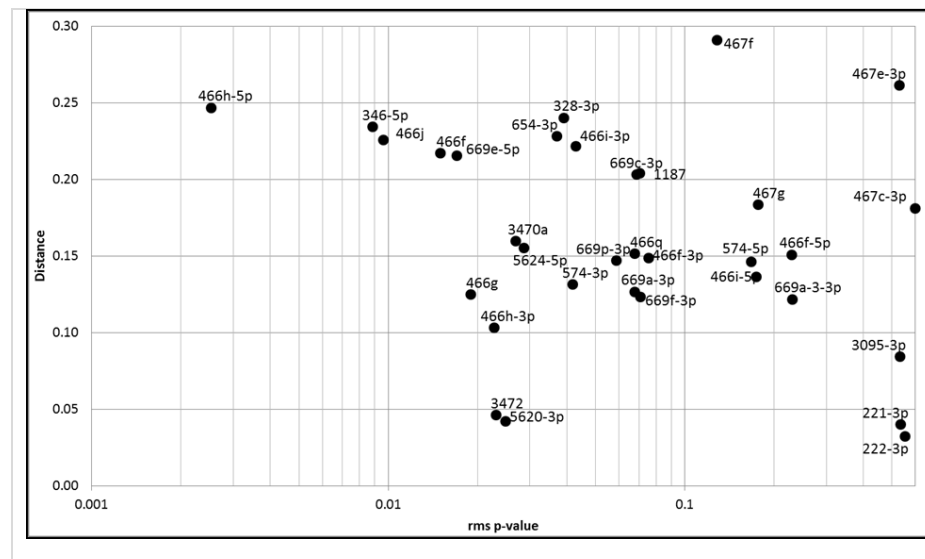


Fig. 8. Sal/Gu-responsive genes that activate RANKL-driven bone resorption. Salubrinal and guanabenz downregulate microRNA such as 466h-5p, 346-5p, 466j, etc.

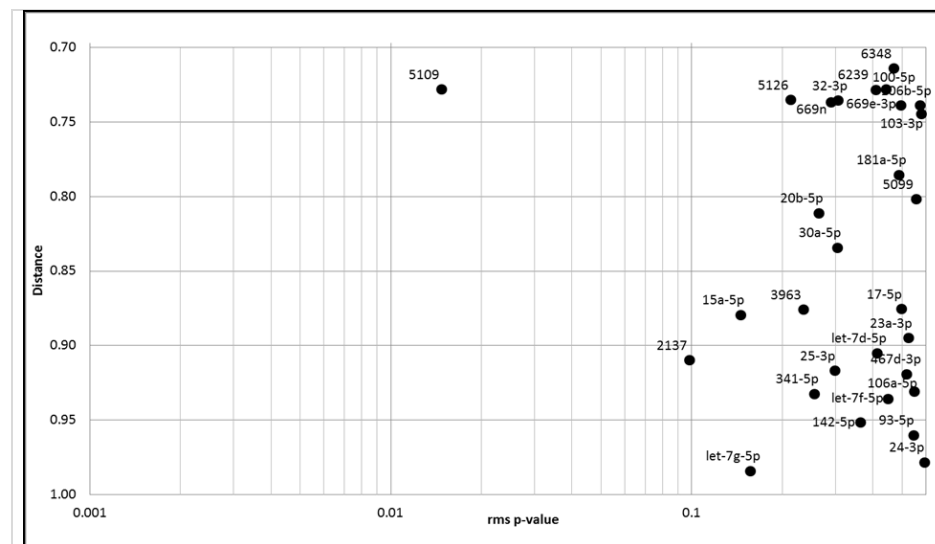


Fig. 9. Sal/Gu-responsive genes that inhibit RANKL-driven bone resorption. Salubrinal and guanabenz upregulate micro RBA such as 5109, 2137, 15a-5p, etc.

Since microRNA binding of the 3' UTR of its target mRNA results in gene silencing, the definition of stimulator and inhibitor for microRNA is different. In the regulation of NFATc1, stimulator microRNAs would ideally silence the genes inhibiting NFATc1 expression. On the other hand, inhibitor microRNAs would silence the genes stimulating NFATc1 or NFATc1 itself. NFATc1 is not the only target gene of interest, and thus all predicted regulators should be taken

into count. It is possible that a single microRNA could interact with multiple mRNAs and each of those mRNAs could have multiple microRNA binding sites. A study on the pathogenesis of liver cancer found that Ddit4 is a possible target of miR-221. In our analysis, miR-221-3p was included as a potential stimulator, and Ddit4 was one of its targets.

Collectively, our working hypothesis for Sal/Gu's action is depicted (**Fig. 10**). One possibility is that Sal/Gu may reduce NFATc1 by activating Ddit4, Zfyve21 or inhibiting miR346-5p, miR466i-3p, miR366f, etc. Alternatively, Sal/Gu may suppress NFATc1 by inhibiting Dscr1, Dusp2, Ptpn22, etc. or activating miR149-3p.

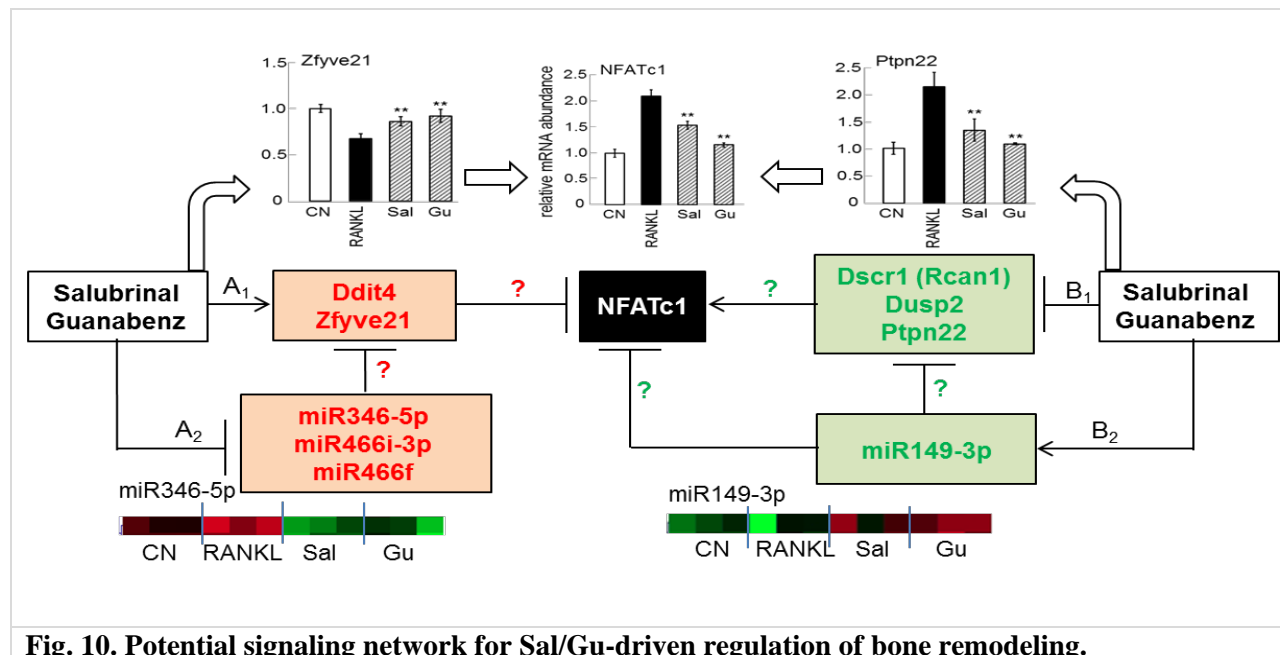


Fig. 10. Potential signaling network for Sal/Gu-driven regulation of bone remodeling.

Task 4: Evaluate the efficacy of salubral on bone fracture healing.

Subtask 4a. Evaluation of the efficacy of salubral on bone fracture healing using s.c. administration.

In the third year, efficacy of salubral on bone fracture healing was examined using a tibia fracture model. Salubral was administered daily via s.c. near the fracture site in the tibia, and X-ray images were collected 1, 2, 3, and 4 weeks after induction of tibia fracture (**Fig. 11**).

With the animal lying supine on the operating table, an intramedullary rod was surgically inserted via the proximal end of the tibia into the medullary

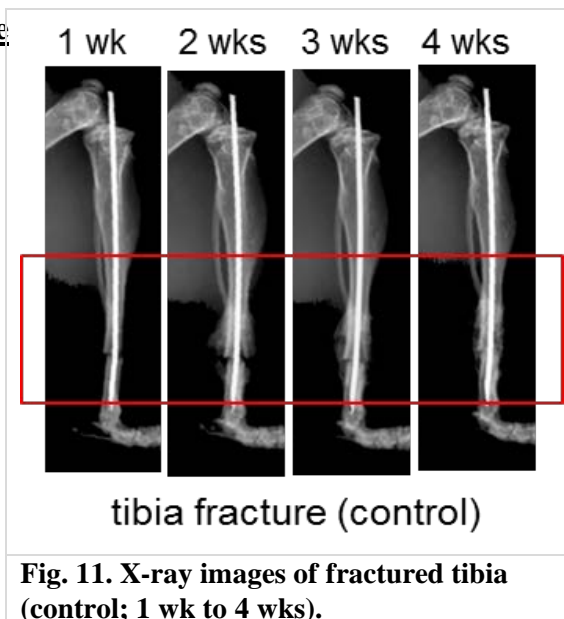


Fig. 11. X-ray images of fractured tibia (control; 1 wk to 4 wks).

canal in order to stabilize the tibia after induction of its fracture. A 200-gram weight was dropped from a height of 30 cm to induce tibia fracture at the site approximately 1/3 of the tibia from the lower end. X-ray imaging was conducted immediately after the operation to confirm fracture induction. A fracture repair process was monitored weekly using radiography (pixRay-100, Bioptics, Phoenix, AZ) for 4 weeks.

Measurements of bone mineral density (BMD) and bone mineral content (BMC) show that administration of salubrinal (1 mg/kg) elevated both BMD and BMC in the entire tibia as well as fracture site (tibial section 3.8 mm long across the fracture site) (**Fig. 12**). The BMD value in the entire tibia was increased from $0.048 \pm 0.0009 \text{ g/cm}^2$ (mean \pm SEM) in the control group to $0.0501 \pm 0.0005 \text{ g/cm}^2$ in the salubrinal-treated group ($p < 0.05$). The BMD value in the fractured callus was also elevated from $0.0501 \pm 0.0013 \text{ g/cm}^2$ in the control group to $0.054 \pm 0.0011 \text{ g/cm}^2$ in the salubrinal-treated group ($p < 0.05$). Furthermore, the BMC value in the entire tibia was increased from $0.0198 \pm 0.0005 \text{ grams}$ in the control group to $0.0211 \pm 0.0003 \text{ grams}$ in the salubrinal-treated group ($p < 0.05$). The BMC value in the fractured callus was also elevated from $0.0053 \pm 0.0002 \text{ grams}$ in the control group to $0.0059 \pm 0.0001 \text{ grams}$ in the salubrinal-treated group ($p < 0.05$).

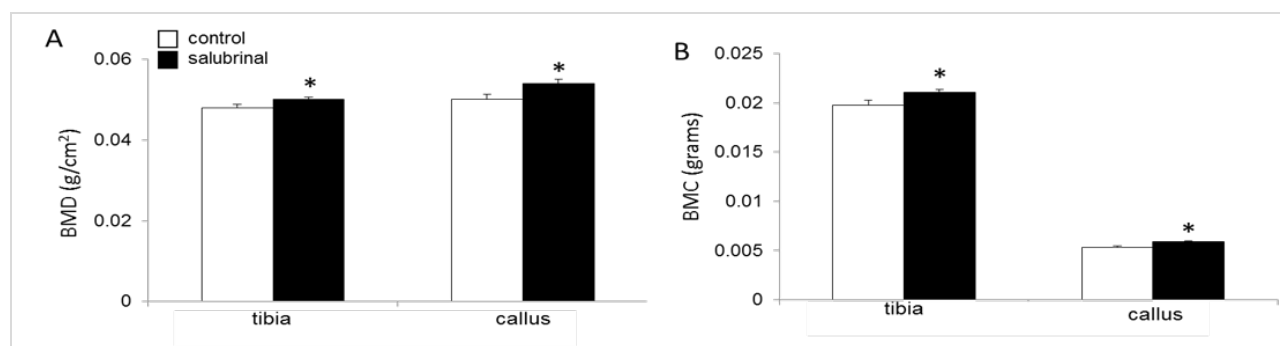


Fig. 12. Comparison of BMD and BMC in the fractured tibia (salubrinal treated samples for 4 weeks). (A) BMD measurements. (B) BMC measurements. Of note, measurements were taken in a boxed region (3.8 mm long).

Subtask 4b. Evaluation of the efficacy of salubrinal on bone fracture healing using local administration.

To locally deliver salubrinal for fracture bone healing, we started designing hydrogel formulations capable of dissolving hydrophobic salubrinal and *in situ* curing at the fracture site. We are examining injectable hydrogel that is formed by reacting eight-arm poly(ethylene glycol) thiol (PEG8SH) and PEG-divinylsulfone (PEGdVS) through a nucleophilic Michael-type conjugation addition reaction. These two macromer components are hydrophilic and can be readily prepared as aqueous solutions. Upon injection through a dual-syringe system, the two macromer solutions mix and become elastic hydrogel within seconds to minutes depending on the macromer molecular weights and concentrations. The formulation used to dissolve salubrinal (up to 1.8mg/mL) is composed of 45% PEG 400, 5% vitamin E d- α -tocopheryl PEG 1000 succinate (TPGS), and 50% phosphate buffer solution. The salubrinal containing formulation is miscible with the hydrogel macromers (PEG8SH and PEGdVS) and therefore can be mixed in

either PEG8SH or PEGdVS solution prior to injection. Following injection, the salubrin- containing hydrogel cures in situ at the bone fracture site. The elastic PEG-based hydrogels will serve both as a structural support and a drug delivery depot to accelerate fracture bone healing.

In the fourth year, we will examine whether the hydrogels can be designed to be degraded in response to proteolytic activity. In particular, PEGdVS can be replaced by protease-sensitive peptides terminated with vinylsulfone moieties. This design will allow cell/protease responsive hydrogel degradation and is expected to further accelerate fracture bone healing.

Task 5: Develop a commercialization plan.

Subtask 5b. Investigate potential partnership options.

In the third year, a small business company, Ossa Biomedical LLC, was established with the objective of commercializing Salubrin and Guanabenz-linked IP. Through Indiana University Research and Technology Cooperation, two key personnel (J.R. Renbarger and Alexander Brethauer) were recruited. Renbarger serves as President and CEO and he is responsible for business strategy and financial support for Ossa Biomedical. Alexander Brethauer serves as CTO and he is responsible for product development, regulatory strategy, and IP strategy. Currently, four patents are being filed for usage of salubrin and its analogs for treatment of osteoporosis and bone fracture:

US patent: Method for treatment of bone diseases and fractures (13/055,399), 3/8/11
US patent: Compositions and methods for treating bone diseases (13/574,105), 3/19/13
US patent: Salubrin driven attenuation of malignant phenotypes of breast cancer cells (14/296,888), 6/5/14
International patent: Compositions and methods for treating bone diseases (PCT/US2014/021682), 3/ 7/14

Subtask 5c. Integrate the results from Aims 2-4, and develop milestones for a clinical trial strategy.

In the third year, we evaluated several options for potential clinical trials including patients with osteoporosis, bone fracture, spinal cord injury, and bone metastasis from breast cancer. In collaboration with Dr. Kathy Miller (Director, Breast Cancer Program, Indiana University School of Medicine), the grant for a clinical trial was received from The Breast Cancer Research Foundation (Title: Repurposing Guanabenz). In this project, we plan to recruit patients with known bone metastases who have been treated with the same skeletal protective agent for at least 3 months. Patients (initial n=15) will undergo baseline assessments, then begin treatment with guanabenz, starting at the lowest FDA approved dose with individual patient dose escalation as tolerated. Limited PK sampling will be obtained to compare exposure with levels associated with activity on these and ongoing preclinical studies. Markers associated with bone turnover will be assessed serially (baseline, 3 and 6 months) to provide initial estimates of biologic activity. Development of the full pilot trial is ongoing. We expect to begin the regulatory approval process with trial activation in November 2014.

B. Plan for the Fourth Year

The fourth year will complete this project by conducting the sub-task 3b (investigation of molecular pathway), the sub-task 4b (analysis of bone fracture healing using local administration of salubrinal), and the sub-task 5c & 5d (data integration, milestones for a clinical trial, and licensing agreement).

C. Key Research Accomplishments

In the third year of this project, the key research accomplishments include the followings:

- The decrease in bone mass and bone formation rate and the increase in osteoblasts and osteocyte apoptosis, which is induced by glucocorticoid at 1.4 mg/kg/day, are abolished by treatment with salubrinal.
- There is a cross talk between salubrinal and alendronate (bisphosphonate) in the responses to GC.
- Genome-wide mRNA expression analysis reveals that both salubrinal and guanabenz downregulate NFATc1 transcription factor, which activates bone resorption, in a novel eIF2 α mediated pathway.
- Genome-wide micro RNA expression analysis shows that salubrinal and guanabenz significantly changes the expression levels of miR346-5p, miR466i-3p, miR466f, and miR149-3p.
- Administration of salubrinal elevates bone formation at the site of tibia fracture by increasing BMD (bone mineral density) and BMC (bone mineral content).

D. Reportable Outcomes

The study in the third year generated the following reportable outcomes.

- An international patent application (compositions and methods for treating bone diseases, PCT/US2014/021682) was filed on March 7, 2014.
- Two conference abstracts were published (Refs. 1 & 2).
- Three peer-reviewed research articles were published (Refs. 3-5). Of note, an additional peer-reviewed journal article is being prepared (Ref. 6).
- A pilot grant from Indiana CTSI (Clinical and translational Sciences Institute) Research Intervention and Scientific Commercialization (Novel pharmacological treatment of osteogenesis imperfecta, \$25,000, 07/01/14 – 06/30/15) was funded for the application of salubrinal and guanabenz in treatment of brittle bone diseases.
- A clinical trial for strengthening bone with guanabenz for patients with bone metastasis is planned to be conducted at Indiana University hospital in November 2014.

E. Conclusion

The study in the third year strongly supported salubrinal's action on the prevention of bone loss by elevating bone formation and inhibiting bone resorption. The observed dual role of salubrinal is unique, and currently no therapeutic drugs provide these dual functions. In the animal experiments for evaluating the effects of salubrinal on glucocorticoid induced osteonecrosis and bone fracture healing, salubrinal presented its anabolic and anti-catabolic effects. In *in vitro* experiments, the molecular mechanisms of salubrinal's action through genome-wide mRNA and micro RNA analyses revealed that salubrinal induces its unique functions through novel targets in bone remodeling, which are mediated by eIF2 α signaling.

F. References

1. Chen AB, Hamamura K, Tanjung N, Yokota H (2014). Principal component analysis of the regulation of osteoclastogenesis by salubrinal and guanabenz. 2014 Annual Meeting of Biomedical Engineering Society, October 22-25, 2014, San Antonio, TX.
2. Sato A, Plotkin LI, Bellido T. Prevention of glucocorticoid induced-apoptosis of osteoblasts and osteocytes by protecting against endoplasmic reticulum (ER) stress. The 35th Annual Meeting of the American Society for Bone and Mineral Research. Minneapolis, MN, USA. *Journal of Bone and Mineral Research* 28 (Supl. 1):S403 201.
3. Chen, A.B., Zhang, P., Yokota, H. (2013). Evaluating treatment of osteoporosis using particle swarm on a bone remodeling mathematical model. *IET Systems Biol.* 7:231-242.
4. Yokota, H., Hamamura, K., Chen, A., Dodge, T.R., Tanjung, N., Abedinpoor, A., Zhang, P. (2013). Effects of salubrinal on development of osteoclasts and osteoblasts from bone marrow-derived cells. *BMC Musculoskeletal Disorders* 14:197.
5. Hamamura, K., Tanjung, N., Yokota, H. (2013). Suppression of osteoclastogenesis through phosphorylation of eukaryotic translation initiation factor 2 alpha. *J. Bone Miner. Metab.* 31:618-628.
6. Zhang P, Hamamura K, Chen A, Dodge T, Wan Q, Na S, Peacock M, Bellido T, Yokota H. Salubrinal attenuates bone loss in a mouse model of postmenopausal osteoporosis (in preparation).

G. Appendix

1. An international patent application (compositions and methods for treating bone diseases, PCT/US2014/021682)
2. Reference 1
3. Reference 5

PCT

REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference
(if desired) (12 characters maximum) 60-11783

Box No. I TITLE OF INVENTION	
COMPOSITIONS AND METHODS FOR TREATING BONE DISEASES	
Box No. II APPLICANT <input type="checkbox"/> This person is also inventor	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) INDIANA UNIVERSITY RESEARCH AND TECHNOLOGY CORPORATION 351 WEST 10TH STREET INDIANAPOLIS, INDIANA 46202 UNITED STATES	Telephone No. Facsimile No. Applicant's registration No. with the Office
E-mail authorization: Marking one of the check-boxes below authorizes the receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority to use the e-mail address indicated in this Box to send, notifications issued in respect of this international application to that e-mail address if those offices are willing to do so. <input type="checkbox"/> as advance copies followed by paper notifications; or <input type="checkbox"/> exclusively in electronic form (no paper notifications will be sent). E-mail address:	
State (that is, country) of nationality: US	State (that is, country) of residence: US
This person is applicant for the purposes of: <input checked="" type="checkbox"/> all designated States <input type="checkbox"/> the States indicated in the Supplemental Box	
Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)	
<input checked="" type="checkbox"/> Further applicants and/or (further) inventors are indicated on a continuation sheet.	
Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE	
The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: <input checked="" type="checkbox"/> agent <input type="checkbox"/> common representative	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) NADER, Bassam S. Brannon Sowers & Cracraft PC 1 North Pennsylvania Street, Suite 800 Indianapolis, Indiana 46204 UNITED STATES	Telephone No. 317-630-2810 Facsimile No. 317-630-2813 Agent's registration No. with the Office 61816
E-mail authorization: Marking one of the check-boxes below authorizes the receiving Office, the International Searching Authority, the International Bureau and the International Preliminary Examining Authority to use the e-mail address indicated in this Box to send, notifications issued in respect of this international application to that e-mail address if those offices are willing to do so. <input type="checkbox"/> as advance copies followed by paper notifications; or <input checked="" type="checkbox"/> exclusively in electronic form (no paper notifications will be sent). E-mail address: docket@bscattorneys.com	
<input type="checkbox"/> Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.	

Box No. III FURTHER APPLICANT(S) AND/OR (FURTHER) INVENTOR(S)

If none of the following sub-boxes is used, this sheet should not be included in the request.

Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

YOKOTA, Hiroki
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Carmel, Indiana 46032
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This person is:

- ☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:
US

State *(that is, country)* of residence:
US

This person is applicant
for the purposes of:



all designated States



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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

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- ☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

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State *(that is, country)* of nationality:
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US

This person is applicant
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- ☐ applicant only
☒ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:
US

State *(that is, country)* of residence:
US

This person is applicant
for the purposes of:



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Name and address: *(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)*

This person is:

- ☐ applicant only
☐ applicant and inventor
☐ inventor only *(If this check-box is marked, do not fill in below.)*

Applicant's registration No. with the Office

State *(that is, country)* of nationality:

State *(that is, country)* of residence:

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☐ Further applicants and/or (further) inventors are indicated on another continuation sheet.

Box No. V DESIGNATIONS

The filing of this request constitutes under Rule 4.9(a) the designation of all Contracting States bound by the PCT on the international filing date, for the grant of every kind of protection available and, where applicable, for the grant of both regional and national patents.

However,

- ☐ DE Germany is not designated for any kind of national protection
- ☐ JP Japan is not designated for any kind of national protection
- ☐ KR Republic of Korea is not designated for any kind of national protection

(The check-boxes above may only be used to exclude (irrevocably) the designations concerned if, at the time of filing or subsequently under Rule 26bis.1, the international application contains in Box No. VI a priority claim to an earlier national application filed in the particular State concerned, in order to avoid the ceasing of the effect, under the national law, of this earlier national application.)

Box No. VI PRIORITY CLAIM AND DOCUMENT

The priority of the following earlier application(s) is hereby claimed:

Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
		national application: country or Member of WTO	regional application: regional Office	international application: receiving Office
item (1) 19/03/2013	61/803,223	US		
item (2)				
item (3)				

☐ Further priority claims are indicated in the Supplemental Box.

Furnishing the priority document(s):

☒ The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) (only if the earlier application(s) was filed with the receiving Office which, for the purposes of this international application, is the receiving Office) identified above as:

☒ all items ☐ item (1) ☐ item (2) ☐ item (3) ☐ other, see Supplemental Box

☐ The International Bureau is requested to obtain from a digital library a certified copy of the earlier application(s) identified above, using, where applicable, the access code(s) indicated below (if the earlier application(s) is available to it from a digital library):

☐ item (1) ☐ item (2) ☐ item (3) ☐ other, see Supplemental Box
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Restore the right of priority: the receiving Office is requested to restore the right of priority for the earlier application(s) identified above or in the Supplemental Box as item(s) (_____). (See also the Notes to Box No. VI; further information *must* be provided to support a request to restore the right of priority.)

Incorporation by reference: where an element of the international application referred to in Article 11(1)(iii)(d) or (e) or a part of the description, claims or drawings referred to in Rule 20.5(a) is not otherwise contained in this international application but is completely contained in an earlier application whose priority is claimed on the date on which one or more elements referred to in Article 11(1)(iii) were first received by the receiving Office, that element or part is, subject to confirmation under Rule 20.6, incorporated by reference in this international application for the purposes of Rule 20.6.

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Choice of International Searching Authority (ISA) (if more than one International Searching Authority is competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):

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<p>This international application contains the following:</p> <p>(a) request form PCT/RO/101 (including any declarations and supplemental sheets) :</p> <p>(b) description (excluding any sequence listing part of the description, see (f), below) :</p> <p>(c) claims :</p> <p>(d) abstract :</p> <p>(e) drawings (if any) :</p> <p>(f) sequence listing part of the description in the form of an image file (e.g. PDF) :</p> <hr/> <p>Total number of sheets (including the sequence listing part of the description if filed as an image file) :</p> <p>(g) sequence listing part of the description</p> <p><input type="checkbox"/> filed in the form of an Annex C/ST.25 text file</p> <p><input type="checkbox"/> WILL BE filed separately on physical data carrier(s), on the same day and in the form of an Annex C/ST.25 text file</p> <p>Indicate type and number of physical data carrier(s)</p>	<p>Number of sheets</p> <p>4</p> <p>33</p> <p>5</p> <p>1</p> <p>15</p> <p>58</p>	<p>This international application is accompanied by the following item(s) (<i>mark the applicable check-boxes below and indicate in right column the number of each item</i>):</p> <p>1. <input type="checkbox"/> fee calculation sheet :</p> <p>2. <input type="checkbox"/> original separate power of attorney :</p> <p>3. <input type="checkbox"/> original general power of attorney :</p> <p>4. <input type="checkbox"/> copy of general power of attorney; reference number: :</p> <p>5. <input type="checkbox"/> priority document(s) identified in Box No. VI as item(s) :</p> <p>6. <input type="checkbox"/> Translation of international application into (<i>language</i>): :</p> <p>7. <input type="checkbox"/> separate indications concerning deposited microorganism or other biological material :</p> <p>8. <input type="checkbox"/> (<i>only where item (f) is marked in the left column</i>) copy of the sequence listing in electronic form (Annex C/ST.25 text file) not forming part of the international application but furnished only for the purposes of international search under Rule 13ter. :</p> <p>9. <input type="checkbox"/> (<i>only where item (f) is marked in the left column</i>) a statement confirming that “the information recorded in electronic form submitted under Rule 13ter is identical to the sequence listing as contained in the international application” as filed via EFS-Web: :</p> <p>10. <input type="checkbox"/> copy of results of earlier search(es) (Rule 12bis.1(a)) :</p> <p>11. <input type="checkbox"/> other (<i>specify</i>): :</p>	<p>Number of items</p>
<p>Figure of the drawings which should accompany the abstract:</p>	<p>Language of filing of the international application:</p> <p>English</p>		

Box No. X SIGNATURE OF APPLICANT, AGENT OR COMMON REPRESENTATIVE

Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).

/Bassam S. Nader/
Bassam S. Nader
Agent Registration No.: 61816
Agent For Applicant

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COMPOSITIONS AND METHODS FOR TREATING BONE DISEASES

GOVERNMENT RIGHTS

This invention was made with government support under DOD W8IXWH-11-1-0716 awarded by the Department of Defense. The government has certain rights in the invention.

TECHNICAL FIELD

The invention described herein pertains to the treatment of bone diseases. In particular, the invention described herein pertains to the treatment of bone diseases responsive to the inhibition of osteoclastogenesis and/or stimulation of osteoblast development and function.

BACKGROUND AND SUMMARY OF THE INVENTION

Osteoblasts and osteoclasts are the two major types of bone cells in bone remodeling. Osteoblasts are bone-forming cells originated from mesenchymal stem cells, while osteoclasts are bone-resorbing cells derived from hematopoietic stem cells. These two types of cells orchestrate a complex remodeling process, in which mineralized bone matrix is degraded by osteoclasts and newly formed by osteoblasts [1, 2]. In order to maintain proper bone mass, exercise and calcium rich diets are recommended. However, a failure of the coordinated action such as in osteoporosis, which is a common form of bone loss prevailing among postmenopausal women, increases risk of bone fracture [3]. In order to develop therapeutic drugs for treatment of osteoporosis, an understanding of signaling pathways that govern osteoclastogenesis – development of pre-osteoclasts (monocyte/macrophage) to multi-nucleated osteoclasts – is required.

Currently, the most common medications, prescribed for preventing bone loss in patients with osteoporosis, are bisphosphonates. Bisphosphonates preferentially bind to calcium in bone and induce apoptosis of osteoclasts [15]. Other medications using neutralizing antibodies targeted to RANKL would block osteoclastogenesis by mimicking OPG's binding to RANKL [16]. RANKL is a cytokine belonging to the tumor necrosis factor family, and is involved in T cell-dependent immune responses as well as differentiation and activation of osteoclasts [9, 10].

A novel target for treating such diseases is the protein complex, eIF2, which is a heterotrimer essential for protein synthesis, and eIF2 α is one of its major components together with eIF2 β and eIF2 γ [4]. In response to various stresses such as oxidation, radiation, and stress to the endoplasmic reticulum that potentially lead to cellular apoptosis, a serine residue of eIF2 α is phosphorylated. This action would initiate a pro-survival program by lowering general

translation efficiency except for a group of genes that includes activating transcription factor 4 (ATF4) [5]. ATF4 is a transcription factor critical for osteoblastogenesis and bone formation [6]. In osteoblasts elevation of phosphorylated eIF2 α (p-eIF2 α) is reported to stimulate the expression of ATF4 [7, 8]. Little is known, however, about potential effects of p-eIF2 α on development of osteoclasts.

In response to various stresses including viral infection, nutrient deprivation, and stress to the endoplasmic reticulum, eukaryotic translation initiation factor 2 alpha (eIF2 α) is phosphorylated to cope with stress induced apoptosis. Although bone cells are sensitive to environmental stresses that alter the phosphorylation level of eIF2 α , little is known about the role of eIF2 α mediated signaling during the development of bone-resorbing osteoclasts. It has been discovered herein that by selectively inhibiting de-phosphorylation of eIF2 α , the effects of phosphorylation of eIF2 α on osteoclastogenesis of RAW264.7 pre-osteoclasts as well as development of MC3T3 E1 osteoblast-like cells are affected. Two illustrative agents, salubrinal and guanabenz, demonstrate that selectively inhibiting de-phosphorylation of eIF2 α , results in stimulated matrix deposition of osteoblasts through upregulation of activating transcription factor 4 (ATF4), reduced expression of nuclear factor of activated T cells c1 (NFATc1) and inhibited differentiation of RAW264.7 cells to multi-nucleated osteoclasts. In contrast, partial silencing of eIF2 α with RNA interference reduced suppression of salubrinal/guanabenz-driven downregulation of NFATc1. It has been observed herein that the elevated phosphorylation level of eIF2 α not only stimulates osteoblastogenesis but also inhibits osteoclastogenesis through regulation of ATF4 and NFATc1, supporting the role of such selective inhibitor of de-phosphorylation of eIF2 α in treating and/or preventing bone loss in osteoporosis, fracture, and other bone diseases.

It has also been discovered herein that the compounds described herein can regulate expression of NFATc1 at a transcriptional level. Without being bound by theory, it is believed herein that the elevation of p-eIF2 α stimulates osteocalcin expression through upregulation of ATF4 in osteoblasts and inhibits TRAP expression via downregulation of NFATc1 in pre-osteoclasts. Silencing eIF2 α with RNA interference reduces suppression of salubrinal/guanabenz-driven downregulation of NFATc1. Thus, the compounds described herein are useful in regulating bone remodeling through eIF2 α -mediated signaling for combating bone loss in osteoporosis, and related diseases.

Osteoblasts and osteoclasts extensively interact through molecular pathways including RANK (receptor activator of nuclear factor kappa-B)/RANKL (RANK ligand)/OPG (osteoprotegerin) signaling [9, 10] and Wnt signaling [11]. It has been discovered herein that

osteoclastogenesis is regulated by signaling molecules that also affect osteoblastogenesis. Furthermore, it has been discovered that osteoclastogenesis is influenced by various stresses such as estrogen deficiency and disuse or unloading [12]. It has also been discovered herein that elevation of p-eIF2 α suppresses differentiation of pre-osteoclasts to multi-nucleated osteoclasts, and that elevation of p-eIF2 α can provide stress-relieving effects on osteoblasts.

In one embodiment, described herein are compounds, compositions, methods, and uses for treating and/or preventing bone loss in osteoporosis, fracture, and other bone diseases. The compounds, compositions, methods, and uses include selective inhibitors de-phosphorylation of eIF2 α , such as salubrinal and guanabenz [13, 14]. It is understood herein that the signaling pathway, mediated by eIF2 α , is not directly linked to known agents for osteoclastogenesis such as calcium binding agents and RANKL.

In another embodiment, pharmaceutical compositions containing one or more of the compounds are also described herein. It is to be understood that the compositions may include other component and/or ingredients, including, but not limited to, other therapeutically active compounds, and/or one or more carriers, diluents, excipients, and the like.

It is appreciated herein that the compounds described herein may be used alone or in combination with other compounds useful for treating bone diseases, including those compounds that may be therapeutically effective by the same or different modes of action. In addition, it is appreciated herein that the compounds described herein may be used in combination with other compounds that are administered to treat other symptoms of bone disease.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Osteogenic effects of salubrinal on MC3T3 E1 (clone 14) osteoblast cells. CN = control, Sal = salubrinal, and Tg = thapsigargin. The double asterisk indicates $p < 0.01$ in comparison to CN. (A) Cell mortality ratio and relative cell numbers. (B) No activation of cleaved caspase 3 by salubrinal. (C) Alizarin red S staining area in response to 5, 10, and 20 μ M salubrinal.

Figure 2. Upregulation of p-eIF2 α , ATF4 and osteocalcin by salubrinal in MC3T3 E1 (clone 14) osteoblast cells in response to 5 μ M salubrinal. CN = control, Sal = salubrinal, and NC = non-specific control siRNA. The double asterisk indicates $p < 0.01$ in comparison to CN or NC. The double dagger indicates with $p < 0.01$ in comparison to the salubrinal-treated NC siRNA cells. (A) Western blot analysis of p-eIF2 α and ATF4. (B) Salubrinal driven elevation of osteocalcin mRNA level. (C) ATF4 level after transfecting siRNA specific to ATF4. (D) Relative mRNA levels of ATF4 in response to RNA interference

with ATF4 siRNA and non-specific control (NC) siRNA. (E) Relative mRNA levels of osteocalcin (OCN). The asterisk is for the comparison to the control with NC siRNA, and the dagger is the comparison between the samples transfected with ATF4 siRNA.

Figure 3. Inhibitory effects of salubrinal on RAW264.7 pre-osteoclasts. CN = control, and Sal = salubrinal. The single and double asterisks indicate $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. (A) Cell mortality ratio. (B) Relative cell numbers. (C) Dose-dependent suppression of RANKL driven activation of osteoclasts by salubrinal. (D) Dose-dependent suppression of TRAP-positive multi-nucleated cells by salubrinal.

Figure 4. Reduction of RANKL-induced NFATc1 expression by salubrinal. Sal = salubrinal. The relative intensity of NFATc1 to β -actin is shown. (A) Expression of NFATc1 (2 days after RANKL administration). (B) Expression of NFATc1 (4 days after RANKL administration).

Figure 5. Effects of salubrinal on mRNA expression levels of NFATc1, c-Fos, TRAP, and OSCAR. CN = control. The single and double asterisks indicate significant decreases with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. The single and double daggers indicate significant increases with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. (A) Messenger RNA levels (2 days after RANKL administration). (B) Messenger RNA levels (4 days after RANKL administration).

Figure 6. Temporal expression profile of p-ERK, p-p38 MAPK, p-NF κ B, p-eIF2 α and NFATc1 in the presence and absence of 20 μ M salubrinal. (A) Western blot analysis of p-ERK, p-p38 MAPK, p-NF κ B, and p-eIF2 α at 15, 30, 60 and 120 min. (B) Western blot analysis of eIF2 α -p and NFATc1. (C) Comparison of the expression level of eIF2 α -p and NFATc1 with and without 20 μ M salubrinal. The normalized level of "1" was defined as the level for the cells that were not treated with RANKL without administration of salubrinal.

Figure 7. Inhibitory effects of guanabenz on development of RAW264.7 pre-osteoclasts. CN = control, and Gu = guanabenz. The single and double asterisks indicate significant decreases with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. (A) Cell mortality ratio. (B) Relative cell numbers. (C) Dose-dependent suppression of RANKL driven activation of osteoclasts by guanabenz. (D) Dose-dependent suppression of TRAP-positive multi-nucleated cells by guanabenz.

Figure 8. Reduction of RANKL-induced NFATc1 expression by guanabenz. CN = control, and Gu = guanabenz. The single and double asterisks indicate significant decreases

with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. (A) Expression of NFATc1 (2 days after RANKL administration). (B) Messenger RNA levels of NFATc1, c-Fos, TRAP, and OSCAR (2 days after RANKL administration).

Figure 9. Temporal expression profile of p-eIF2 α and NFATc1 in the presence and absence of 20 μ M guanabenz. Gu = guanabenz. (A) Western blot analysis of eIF2 α -p and NFATc1. (B) Comparison of the expression level of eIF2 α -p and NFATc1 with and without 20 μ M guanabenz. The normalized level of “1” was defined as the level for the cells that were not treated with RANKL without administration of guanabenz.

Figure 10. Reduction in salubrinal/guanabenz driven suppression of NFATc1 expression by RNA interference specific for eIF2 α . Sal = salubrinal, Gu = guanabenz, and NC = non-specific control siRNA. The single and double asterisks indicate significant changes to the RANKL-treated NC siRNA cells with $p < 0.05$ and $p < 0.01$, respectively. The single and double daggers indicate significant changes to the salubrinal or guanabenz-treated NC siRNA cells with $p < 0.05$ and $p < 0.01$, respectively. (A) eIF2 α level after transfecting siRNA specific to eIF2 α . (B) Western blot analysis of p-NF κ B and NFATc1. (C) Comparison of the expression level of NFATc1 between control siRNA and eIF2 α siRNA.

Figure 11 shows, though without being bound by theory, an illustrative mechanism of eIF2 α signaling on osteoclastogenesis through NFATc1.

Figure S1. Osteogenic effects of salubrinal on MC3T3 E1 osteoblast cells. CN = control, and Sal = salubrinal. The double asterisk indicates $p < 0.01$ in comparison to CN. (A) Cell mortality ratio in response to 0.1 – 30 μ M salubrinal. (B) Relative cell numbers in response to 0.1 – 30 μ M salubrinal. (C) Alizarin red S staining area in response to 0.1, 0.5, 1, 5, 10, and 20 μ M salubrinal.

Figure S2. Upregulation of p-eIF2 α , ATF4 and osteocalcin by salubrinal in MC3T3 E1 osteoblast cells. CN = control, and Sal = salubrinal. The single and double asterisks indicate $p < 0.05$ and $p < 0.01$ in comparison to CN, respectively. (A) Western blot analysis of p-eIF2 α and ATF4. (B) Salubrinal-driven elevation of the levels of ATF4 mRNA and osteocalcin mRNA.

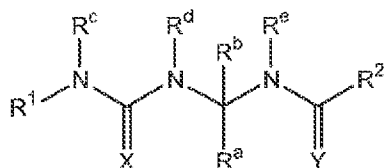
Figure S3. Osteogenic effects of guanabenz on MC3T3 E1 osteoblast cells. CN = control, and Gu = guanabenz. The single and double asterisks indicate $p < 0.05$ and $p < 0.01$ in comparison to CN, respectively. (A) Cell mortality ratio. (B) Relative cell numbers. (C) Western blot analysis of p-eIF2 α and ATF4. (D) Guanabenz-driven elevation of the osteocalcin mRNA level.

Figure S4. No significant effects of salubrinal and guanabenz on cell mortality,

cell numbers, and expression of NFATc1 and TRAP in RAW264.7 cells in the absence of RANKL administration. CN = control, Sal = salubrinal, and Gu = guanabenz. (A) Cell mortality ratio. (B) Relative cell numbers. (C) Expression of NFATc1. (D) Relative mRNA levels of NFATc1 and TRAP.

5 DETAILED DESCRIPTION

In one embodiment, selective inhibitors of de-phosphorylation of eIF2 α are described herein for treating and/or preventing bone loss in osteoporosis, fracture, and other bone diseases. Illustrative inhibitors include compounds of the formula



10 or a pharmaceutically acceptable salt thereof, wherein:

X is O or S;

Y is O or S;

R¹ is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, or heteroaryl, each of which is optionally substituted;

15 R² is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted;

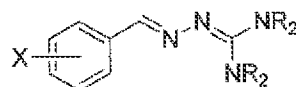
R³ is optionally substituted alkyl;

Rᵇ is H or optionally substituted C₁-C₆ alkyl;

20 Rᶜ, Rᵈ, and Rᵉ are each independently selected from the group consisting of H, optionally substituted C₁-C₆ alkyl, acyl, or a prodrug capable of releasing the attached nitrogen in vivo .

It is to be understood that the various genera and subgenera of each of X, Y, R¹, R², R³, Rᵇ, Rᶜ, Rᵈ, and Rᵉ are described herein. It is to be understood that all possible combinations of the various genera and subgenera of each of X, Y, R¹, R², R³, Rᵇ, Rᶜ, Rᵈ, and Rᵉ are described herein represent additional illustrative embodiments of compounds of the invention described herein. It is to be further understood that each of those additional illustrative embodiments of compounds may be used in any of the compositions, methods, and/or uses described herein.

30 Further illustrative inhibitors include compounds of the formula



where

X represents five substituents each independently selected from hydrogen, halo, hydroxy, amino, thio, carboxylate or a derivative thereof, sulfinyl or a derivative thereof, sulfonyl or a derivative thereof, phosphinyl or a derivative thereof, or phosphonyl or a derivative thereof, or alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, heteroalkyl, heteroalkenyl, cycloheteroalkyl, cycloheteroalkenyl, aryl, heteroaryl, arylalkyl, or heteroarylalkyl, each of which is optionally substituted; and

R is independently selected in each instance from hydrogen, hydroxy, amino, carboxylate and derivatives thereof, sulfonyl and derivatives thereof, and alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, heteroalkyl, heteroalkenyl, cycloheteroalkyl, cycloheteroalkenyl, aryl, heteroaryl, arylalkyl, and heteroarylalkyl, each of which is optionally substituted, and nitrogen prodrug forming groups.

MC3T3 E1 osteoblast-like cells [17] and RAW264.7 cells [18] are employed to evaluate osteoblastogenesis and osteoclastogenesis, respectively. In the presence and absence of salubrinal and guanabenz, MC3T3 E1 cells are cultured in an osteogenic medium for evaluation of matrix deposition, while RAW264.7 cells in an osteoclast differentiation medium for evaluation of multi-nucleation. Alizarin Red S staining is performed to evaluate osteoblast mineralization for MC3T3 E1 cells, and TRAP staining is conducted to determine multi-nucleated osteoclasts proliferation for RAW264.7 cells. To analyze molecular signaling pathways, quantitative real-time PCR and Western blot analysis are conducted. The mRNA levels of ATF4, osteocalcin, c-Fos [19], tartrate-resistant acid phosphatase (TRAP) [20], and osteoclast-associated receptor (OSCAR) [21] are determined. The protein expression levels of eIF2 α , ATF4, and nuclear factor of activated T cells c1 (NFATc1) [22] are also determined. NFATc1 is a transcription factor, which is reportedly important for development and activation of osteoclasts in response to RANKL. RNA interference using siRNA specific to ATF4 and eIF2 α is conducted to evaluate the role of ATF4 in osteoblastogenesis and eIF2 α in osteoclastogenesis.

Without being bound by theory it is believed herein that the compounds described herein are efficacious, at least in part, due to the ability to inhibit the differentiation of RAW264.7 pre-osteoclasts to multi-nucleated osteoclasts. Without being bound by theory it is believed herein that the compounds described herein are efficacious, at least in part, due to the ability to block de-phosphorylation of eIF2 α and elevate the level of p-eIF2 α . It has been discovered herein that the growth area, covered by multi-nucleated cells, is significantly reduced by salubrinal and guanabenz in a dose dependent manner. Partially silencing eIF2 α

using RNA interference significantly suppressed salubrinal/guanabenz-driven reduction of NFATc1 expression. Together with the stimulated development of MC3T3 E1 osteoblasts by an increase in ATF4 expression, the results herein support the conclusion that eIF2 α mediated signaling plays a physiological role in osteoclastogenesis and osteoblastogenesis, and can be used in treating osteoporosis, fractures, and other bone diseases and disorders.

It is understood herein that both salubrinal and guanabenz interact with PP1 and inhibit its activity of de-phosphorylating p-eIF2 α . Furtch, guanabenz is reported to bind to PP1R15A subunit [14], while the exact binding site of salubrinal is not known. Guanabenz is also known as an α_2 -adrenergic receptor agonist and used to treat hypertension [24]. Without being bound by theory, it is believed herein that the observed stimulation of osteoblastogenesis as well as attenuation of osteoclastogenesis by both agents strongly indicates that eIF2 α -mediated signaling is central to regulation of ATF4 and NFATc1. This belief is also supported by the salubrinal-driven alterations in the mRNA levels of osteocalcin and TRAP, which are representative in development of osteoblasts and osteoclasts, respectively. Osteocalcin is synthesized solely by osteoblasts for matrix mineralization and calcium homeostasis [25], while TRAP is highly expressed in osteoclasts and its overexpression has been observed to cause bone loss in transgenic mice [26].

The elevation of p-eIF2 α has been discovered herein to enhance the development of osteoblasts and mineralization of extracellular matrix. In response to various stresses such as oxidation, radiation, and stress to the endoplasmic reticulum, cells undergo either survival or apoptotic pathway [27]. As part of a pro-survival program, the level of p-eIF2 α is raised followed by diminished translational efficiency to all proteins except for a limited group including ATF4 [5]. Salubrinal's action mimics the induction of pro-survival program without imposing damaging stresses, which result in the upregulation of ATF4 without inducing apoptosis. Since ATF4 is a transcription factor critical for osteoblastogenesis and bone formation, the effects of administration of salubrinal and guanabenz on the mRNA level of osteocalcin as well as the mineralization of extracellular matrix was examined. Silencing ATF4 using RNA interference significantly suppressed salubrinal-driven upregulation of osteocalcin expression. Thus, the result here is consistent with the previously reported role of salubrinal that stimulates new bone formation in the healing of bone wound [8].

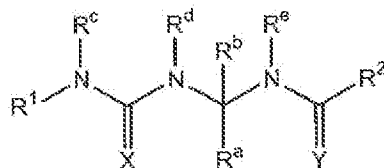
Without being bound by theory, a schematic diagram illustrating a proposed pathway of eIF2 α -mediated signaling in osteoblastogenesis and osteoclastogenesis is presented (Fig. 11). Through inhibition of de-phosphorylation of eIF2 α , salubrinal and guanabenz are capable of enhancing bone formation by activating ATF4, as well as reducing bone resorption

by down-regulating NFATc1. Osteoclastogenesis is a multicomponent developmental process, in which active interactions take place between osteoblasts and osteoclasts. In the RANK/RANKL/OPG signaling pathway, for instance, osteoblasts provide RANKL that stimulates osteoclastogenesis. Since binding of RANKL to RANK is known to activate MAPKs and NFκB [28, 29], the potential role of ERK, p38, and NFκB in the eIF2α-mediated signaling. The levels of p-ERK, p-p38 MAPK, and p-NFκB together with p-eIF2α were evaluated by measuring the response to administration of 20 μM salubrinal. However, no detectable changes in the levels of their phosphorylated form were observed. Without being bound by theory, it is believed herein that salubrinal may activate transcription factors such as MafB (V-maf musculoaponeurotic fibrosarcoma oncogene homolog B), IRF8 (interferon regulatory factor 8), and Bcl6 (B-cell lymphoma 6), which are known to be inhibitors of NFATc1 [30-32]. Alternatively, microRNA and epigenetic changes such as histone modification regulate expression of NFATc1 might be involved [33, 34]. For instance, H3K27 demethylase is reported to demethylate the site of H3K27me3 of NFATc1 and stimulate RANKL-induced osteoclastogenesis [34].

Several illustrative embodiments of the invention are described by the following enumerated clauses:

1. A method for treating osteoporosis, fracture, or a bone defect in a host animal, the method comprising the step of administering to the host animal a therapeutically effective amount of one or more compounds capable of selectively inhibiting de-phosphorylation of eIF2α; where the amount is capable of (a) stimulating matrix deposition of osteoblasts, (b) upregulating activating transcription factor 4 (ATF4), (c) reducing expression of nuclear factor of activated T cells c1 (NFATc1), (d) inhibiting differentiation of RAW264.7 cells to multi-nucleated osteoclasts, (e) stimulating osteoblastogenesis, or (f) inhibiting osteoclastogenesis, or a combination of the foregoing, or a pharmaceutically acceptable composition comprising the one or more compounds.

2. The method clause 1 wherein at least one compound is of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X is O or S;

Y is O or S;

R¹ is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, or heteroaryl, each of which

is optionally substituted;

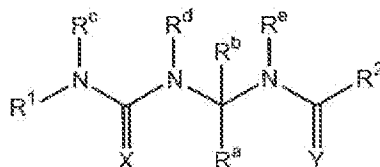
R^2 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted;

5 R^a is optionally substituted alkyl;

R^b is H or optionally substituted C_1 - C_6 alkyl;

R^c , R^d , and R^e are each independently selected from the group consisting of H, optionally substituted C_1 - C_6 alkyl, acyl, or a prodrug capable of releasing the attached nitrogen in vivo.

10 3. The method clause 1 or 2 wherein at least one compound is of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X is O or S;

15 Y is O or S;

R^1 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, or heteroaryl, each of which is optionally substituted;

R^2 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted;

20 R^a is optionally substituted alkyl;

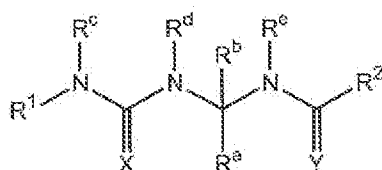
R^b is H or optionally substituted C_1 - C_6 alkyl;

R^c , R^d , and R^e are each independently selected from the group consisting of H, optionally substituted C_1 - C_6 alkyl, acyl, or a prodrug capable of releasing the attached nitrogen in vivo;

25

or a composition thereof further comprising one or more carriers, diluents, or excipients, or a combination thereof.

4. The method of any one of the preceding clauses wherein at least one compound is of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X is O or S;

Y is O or S;

5 R^1 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, or heteroaryl, each of which is optionally substituted;

R^2 is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted;

10 R^a is optionally substituted alkyl;

R^b is H or optionally substituted C_1 - C_6 alkyl;

R^c , R^d , and R^e are each independently selected from the group consisting of H, optionally substituted C_1 - C_6 alkyl, acyl, or a prodrug capable of releasing the attached nitrogen in vivo.

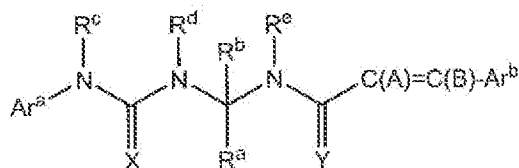
15 5. The method of any one of the preceding clauses wherein R^1 is alkyl, aryl, or heteroaryl, each of which is optionally substituted.

6. The method of any one of the preceding clauses wherein R^1 is aryl or heteroaryl, each of which is optionally substituted.

20 7. The method of any one of the preceding clauses wherein R^2 is alkenyl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted.

8. The method of any one of the preceding clauses wherein R^2 is alkenyl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted.

25 9. The method of any one of the preceding clauses wherein at least one compound is a compound of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X and Y are independently O or S;

Ar^a and Ar^b are independently aryl or heteroaryl, each of which is optionally substituted;

R^a is optionally substituted alkyl;

R^b is H or optionally substituted C_1 - C_6 alkyl;

5 R^c , R^d , and R^e are each independently selected from the group consisting of H, optionally substituted C_1 - C_6 alkyl, acyl, and a prodrug capable of releasing the attached nitrogen in vivo to form the corresponding H or salt derivative thereof;

and A and B are independently H, or optionally substituted C_1 - C_6 alkyl

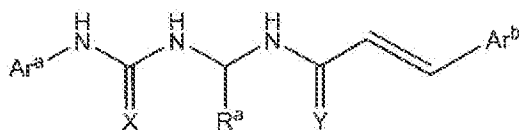
10. The method of clause 9 wherein the aryl is a bicyclic aryl.

11. The method of clause 9 or 10 wherein the heteroaryl is a bicyclic heteroaryl.

12. The method of any one of clauses 9 to 11 wherein the aryl or heteroaryl is optionally substituted with one or more substituents selected from the group consisting of halo, nitrile, or optionally substituted C_1 - C_6 alkyl, C_1 - C_6 alkoxy, C_1 - C_6 haloalkyl, C_1 - C_6 haloalkoxy, aryl, aryloxy, heterocyclyl, heterocyclyloxy, fused aryl, and fused heterocyclyl.

13. The method of any one of clauses 9 to 12 wherein the alkenyl has an E geometry.

14. The method of any one of the preceding clauses wherein at least one compound is of the formula



or a pharmaceutically acceptable salt thereof, wherein,

X is O or S;

Y is O or S;

25 Ar^a and Ar^b are independently aryl or heteroaryl, each of which is optionally substituted; and

R^a is optionally substituted alkyl.

15. The method of clause 14 wherein the aryl is a bicyclic aryl.

16. The method of clause 14 or 15 wherein Ar^a is a bicyclic heteroaryl.

17. The method of any one of clauses 14 to 16 wherein the aryl or heteroaryl is optionally substituted with one or more substituents selected from the group consisting of halo, nitrile, or optionally substituted C_1 - C_6 alkyl, C_1 - C_6 alkoxy, C_1 - C_6 haloalkyl, C_1 - C_6 haloalkoxy, aryl, aryloxy, heterocyclyl, heterocyclyloxy, fused aryl, and fused heterocyclyl.

18. The method of any one of clauses 14 to 17 wherein R^a is haloalkyl.

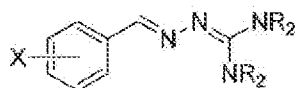
19. The method of any one of clauses 14 to 18 wherein R^a is not trifluoromethyl.

20. The method of any one of clauses 14 to 19 wherein R^a is haloalkyl, where halo is selected from the group consisting of chloro and bromo, and combinations thereof.

21. The method of any one of clauses 14 to 20 wherein R^c , R^d , and R^e are each independently selected from the group consisting of H, optionally substituted C_1 - C_6 alkyl, and acyl.

22. The method of any one of the preceding clauses wherein at least one compound is salubrinol, or an analog or a derivative thereof, or a pharmaceutically acceptable salt of the foregoing.

23. The method of any one of the preceding clauses wherein at least one compound is of the formula



or a pharmaceutically acceptable salt thereof, wherein

X represents five substituents each independently selected from hydrogen, halo, hydroxy and derivatives thereof, amino and derivatives thereof, thio and derivatives thereof, carboxylate or a derivative thereof, sulfinyl or a derivative thereof, sulfonyl or a derivative thereof, phosphinyl or a derivative thereof, or phosphonyl or a derivative thereof, or alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, heteroalkyl, heteroalkenyl, cycloheteroalkyl, cycloheteroalkenyl, aryl, heteroaryl, arylalkyl, or heteroarylalkyl, each of which is optionally substituted; and

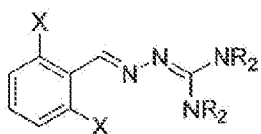
R is independently selected in each instance from hydrogen, hydroxy and derivatives thereof, amino and derivatives thereof, carboxylate and derivatives thereof, sulfonyl and derivatives thereof, and alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, heteroalkyl, heteroalkenyl, cycloheteroalkyl, cycloheteroalkenyl, aryl, heteroaryl, arylalkyl, and heteroarylalkyl, each of which is optionally substituted, and nitrogen prodrug forming groups.

24. The method of clause 23 wherein at least one X is halo, such as chloro.

25. The method of clause 23 wherein at least one X is optionally substituted alkyl, such as methyl or trifluoromethyl.

26. The method of clause 23 wherein at least one X is optionally substituted alkoxy, such as methoxy or trifluoromethoxy.

27. The method of any one of the preceding clauses wherein at least one compound is of the formula



or a pharmaceutically acceptable salt thereof

28. The method of clause 27 wherein each X is halo.
29. The method of clause 27 wherein each X is chloro.
- 5 30. The method of any one of clauses 27 to 29 wherein each R is hydrogen
31. The method of any one of clauses 27 to 30 wherein at least one compound is guanabenz or a pharmaceutically acceptable salt thereof.
32. The method of any one of the preceding clauses wherein the composition further comprises one or more carriers, diluents, or excipients, or a combination thereof.
- 10 33. The method of any one of the preceding clauses wherein the host animal is a human.

The compounds described herein may contain one or more chiral centers, or may otherwise be capable of existing as multiple stereoisomers. It is to be understood that in one embodiment, the invention described herein is not limited to any particular stereochemical requirement, and that the compounds, and compositions, methods, uses, and medicaments that include them may be optically pure, or may be any of a variety of stereoisomeric mixtures, including racemic and other mixtures of enantiomers, other mixtures of diastereomers, and the like. It is also to be understood that such mixtures of stereoisomers may include a single stereochemical configuration at one or more chiral centers, while including mixtures of stereochemical configuration at one or more other chiral centers.

Similarly, the compounds described herein may include geometric centers, such as cis, trans, E, and Z double bonds. It is to be understood that in another embodiment, the invention described herein is not limited to any particular geometric isomer requirement, and that the compounds, and compositions, methods, uses, and medicaments that include them may be pure, or may be any of a variety of geometric isomer mixtures. It is also to be understood that such mixtures of geometric isomers may include a single configuration at one or more double bonds, while including mixtures of geometry at one or more other double bonds.

As used herein, the term "alkyl" includes a chain of carbon atoms, which is optionally branched. As used herein, the term "alkenyl" and "alkynyl" includes a chain of carbon atoms, which is optionally branched, and includes at least one double bond or triple bond, respectively. It is to be understood that alkynyl may also include one or more double bonds. It is to be further understood that in certain embodiments, alkyl is advantageously of limited length, including C₁-C₂₄, C₁-C₁₂, C₁-C₈, C₁-C₆, and C₁-C₄. Illustratively, such

particularly limited length alkyl groups, including C₁-C₈, C₁-C₆, and C₁-C₄ may be referred to as lower alkyl. It is to be further understood that in certain embodiments alkenyl and/or alkynyl may each be advantageously of limited length, including C₂-C₂₄, C₂-C₁₂, C₂-C₈, C₂-C₆, and C₂-C₄. Illustratively, such particularly limited length alkenyl and/or alkynyl groups, including C₂-C₈, C₂-C₆, and C₂-C₄ may be referred to as lower alkenyl and/or alkynyl. It is appreciated herein that shorter alkyl, alkenyl, and/or alkynyl groups may add less lipophilicity to the compound and accordingly will have different pharmacokinetic behavior. In embodiments of the invention described herein, it is to be understood, in each case, that the recitation of alkyl refers to alkyl as defined herein, and optionally lower alkyl. In embodiments of the invention described herein, it is to be understood, in each case, that the recitation of alkenyl refers to alkenyl as defined herein, and optionally lower alkenyl. In embodiments of the invention described herein, it is to be understood, in each case, that the recitation of alkynyl refers to alkynyl as defined herein, and optionally lower alkynyl. Illustrative alkyl, alkenyl, and alkynyl groups are, but not limited to, methyl, ethyl, n-propyl, isopropyl, n-butyl, isobutyl, sec-butyl, tert-butyl, pentyl, 2-pentyl, 3-pentyl, neopentyl, hexyl, heptyl, octyl, and the like, and the corresponding groups containing one or more double and/or triple bonds, or a combination thereof.

As used herein, the term "alkylene" includes a divalent chain of carbon atoms, which is optionally branched. As used herein, the term "alkenylene" and "alkynylene" includes a divalent chain of carbon atoms, which is optionally branched, and includes at least one double bond or triple bond, respectively. It is to be understood that alkynylene may also include one or more double bonds. It is to be further understood that in certain embodiments, alkylene is advantageously of limited length, including C₁-C₂₄, C₁-C₁₂, C₁-C₈, C₁-C₆, and C₁-C₄. Illustratively, such particularly limited length alkylene groups, including C₁-C₈, C₁-C₆, and C₁-C₄ may be referred to as lower alkylene. It is to be further understood that in certain embodiments alkenylene and/or alkynylene may each be advantageously of limited length, including C₂-C₂₄, C₂-C₁₂, C₂-C₈, C₂-C₆, and C₂-C₄. Illustratively, such particularly limited length alkenylene and/or alkynylene groups, including C₂-C₈, C₂-C₆, and C₂-C₄ may be referred to as lower alkenylene and/or alkynylene. It is appreciated herein that shorter alkylene, alkenylene, and/or alkynylene groups may add less lipophilicity to the compound and accordingly will have different pharmacokinetic behavior. In embodiments of the invention described herein, it is to be understood, in each case, that the recitation of alkylene, alkenylene, and alkynylene refers to alkylene, alkenylene, and alkynylene as defined herein, and optionally lower alkylene, alkenylene, and alkynylene. Illustrative alkyl groups are, but not limited to, methylene, ethylene, n-propylene, isopropylene, n-butylene, isobutylene, sec-butylene,

pentylene, 1,2-pentylene, 1,3-pentylene, hexylene, heptylene, octylene, and the like.

As used herein, the term "linker" includes is a chain of atoms that connects two or more functional parts of a molecule to form a conjugate. Illustratively, the chain of atoms is selected from C, N, O, S, Si, and P, or C, N, O, S, and P, C, N, O, and S. The chain of atoms
5 covalently connects different functional capabilities of the conjugate, such as binding ligands, drugs, diagnostic agents, imaging agents, and the like. The linker may have a wide variety of lengths, such as in the range from about 2 to about 100 atoms in the contiguous backbone. The atoms used in forming the linker may be combined in all chemically relevant ways, such as chains of carbon atoms forming alkylene, alkenylene, and alkynylene groups, and the like;
10 chains of carbon and oxygen atoms forming ethers, polyoxyalkylene groups, or when combined with carbonyl groups forming esters and carbonates, and the like; chains of carbon and nitrogen atoms forming amines, imines, polyamines, hydrazines, hydrazones, or when combined with carbonyl groups forming amides, ureas, semicarbazides, carbazides, and the like; chains of carbon, nitrogen, and oxygen atoms forming alkoxyamines, alkoxyamines, or when combined
15 with carbonyl groups forming urethanes, amino acids, acyloxyamines, hydroxamic acids, and the like; and many others. In addition, it is to be understood that the atoms forming the chain in each of the foregoing illustrative embodiments may be either saturated or unsaturated, thus forming single, double, or triple bonds, such that for example, alkanes, alkenes, alkynes, imines, and the like may be radicals that are included in the linker. In addition, it is to be understood
20 that the atoms forming the linker may also be cyclized upon each other or be part of cyclic structure to form divalent cyclic structures that form the linker, including cyclo alkanes, cyclic ethers, cyclic amines, and other heterocycles, arylenes, heteroarylenes, and the like in the linker. In this latter arrangement, it is to be understood that the linker length may be defined by any pathway through the one or more cyclic structures. Illustratively, the linker length is defined
25 by the shortest pathway through the each one of the cyclic structures. It is to be understood that the linkers may be optionally substituted at any one or more of the open valences along the chain of atoms, such as optional substituents on any of the carbon, nitrogen, silicon, or phosphorus atoms. It is also to be understood that the linker may connect the two or more functional parts of a molecule to form a conjugate at any open valence, and it is not necessary
30 that any of the two or more functional parts of a molecule forming the conjugate are attached at any apparent end of the linker.

As used herein, the term "cycloalkyl" includes a chain of carbon atoms, which is optionally branched, where at least a portion of the chain is cyclic. It is to be understood that cycloalkylalkyl is a subset of cycloalkyl. It is to be understood that cycloalkyl may be
35 polycyclic. Illustrative cycloalkyl include, but are not limited to, cyclopropyl, cyclopentyl,

cyclohexyl, 2-methylcyclopropyl, cyclopentyleth-2-yl, adamantyl, and the like. As used herein, the term "cycloalkenyl" includes a chain of carbon atoms, which is optionally branched, and includes at least one double bond, where at least a portion of the chain is cyclic. It is to be understood that the one or more double bonds may be in the cyclic portion of cycloalkenyl and/or the non-cyclic portion of cycloalkenyl. It is to be understood that cycloalkenylalkyl and cycloalkylalkenyl are each subsets of cycloalkenyl. It is to be understood that cycloalkyl may be polycyclic. Illustrative cycloalkenyl include, but are not limited to, cyclopentenyl, cyclohexylethen-2-yl, cycloheptenylpropenyl, and the like. It is to be further understood that chain forming cycloalkyl and/or cycloalkenyl is advantageously of limited length, including C₃-C₂₄, C₃-C₁₂, C₃-C₈, C₃-C₆, and C₅-C₆. It is appreciated herein that shorter alkyl and/or alkenyl chains forming cycloalkyl and/or cycloalkenyl, respectively, may add less lipophilicity to the compound and accordingly will have different pharmacokinetic behavior.

As used herein, the term "heteroalkyl" includes a chain of atoms that includes both carbon and at least one heteroatom, and is optionally branched. Illustrative heteroatoms include nitrogen, oxygen, and sulfur. In certain variations, illustrative heteroatoms also include phosphorus, and selenium. As used herein, the term "cycloheteroalkyl" including heterocyclyl and heterocycle, includes a chain of atoms that includes both carbon and at least one heteroatom, such as heteroalkyl, and is optionally branched, where at least a portion of the chain is cyclic. Illustrative heteroatoms include nitrogen, oxygen, and sulfur. In certain variations, illustrative heteroatoms also include phosphorus, and selenium. Illustrative cycloheteroalkyl include, but are not limited to, tetrahydrofuryl, pyrrolidinyl, tetrahydropyranlyl, piperidinyl, morpholinyl, piperazinyl, homopiperazinyl, quinuclidinyl, and the like.

As used herein, the term "aryl" includes monocyclic and polycyclic aromatic carbocyclic groups, each of which may be optionally substituted. Illustrative aromatic carbocyclic groups described herein include, but are not limited to, phenyl, naphthyl, and the like. As used herein, the term "heteroaryl" includes aromatic heterocyclic groups, each of which may be optionally substituted. Illustrative aromatic heterocyclic groups include, but are not limited to, pyridinyl, pyrimidinyl, pyrazinyl, triazinyl, tetrazinyl, quinolinyl, quinazolinyl, quinoxalinyl, thienyl, pyrazolyl, imidazolyl, oxazolyl, thiazolyl, isoxazolyl, isothiazolyl, oxadiazolyl, thiadiazolyl, triazolyl, benzimidazolyl, benzoxazolyl, benzthiazolyl, benzisoxazolyl, benzisothiazolyl, and the like.

As used herein, the term "amino" includes the group NH₂, alkylamino, and dialkylamino, where the two alkyl groups in dialkylamino may be the same or different, i.e. alkylalkylamino. Illustratively, amino includes methylamino, ethylamino, dimethylamino, methylethylamino, and the like. In addition, it is to be understood that when amino modifies or

is modified by another term, such as aminoalkyl, or acylamino, the above variations of the term amino are included therein. Illustratively, aminoalkyl includes H₂N-alkyl, methylaminoalkyl, ethylaminoalkyl, dimethylaminoalkyl, methylethylaminoalkyl, and the like. Illustratively, acylamino includes acylmethylamino, acylethylamino, and the like.

5 As used herein, the term "amino and derivatives thereof" includes amino as described herein, and alkylamino, alkenylamino, alkynylamino, heteroalkylamino, heteroalkenylamino, heteroalkynylamino, cycloalkylamino, cycloalkenylamino, cycloheteroalkylamino, cycloheteroalkenylamino, arylamino, arylalkylamino, arylalkenylamino, arylalkynylamino, heteroarylamino, heteroarylalkylamino, 10 heteroarylalkenylamino, heteroarylalkynylamino, acylamino, and the like, each of which is optionally substituted. The term "amino derivative" also includes urea, carbamate, and the like.

As used herein, the term "hydroxy and derivatives thereof" includes OH, and alkyloxy, alkenyloxy, alkynyloxy, heteroalkyloxy, heteroalkenyloxy, heteroalkynyloxy, cycloalkyloxy, cycloalkenyloxy, cycloheteroalkyloxy, cycloheteroalkenyloxy, aryloxy, 15 arylalkyloxy, arylalkenyloxy, arylalkynyloxy, heteroaryloxy, heteroarylalkyloxy, heteroarylalkenyloxy, heteroarylalkynyloxy, acyloxy, and the like, each of which is optionally substituted. The term "hydroxy derivative" also includes carbamate, and the like.

As used herein, the term "thio and derivatives thereof" includes SH, and alkylthio, alkenylthio, alkynylthio, heteroalkylthio, heteroalkenylthio, heteroalkynylthio, 20 cycloalkylthio, cycloalkenylthio, cycloheteroalkylthio, cycloheteroalkenylthio, arylthio, arylalkylthio, arylalkenylthio, arylalkynylthio, heteroarylthio, heteroarylalkylthio, heteroarylalkenylthio, heteroarylalkynylthio, acylthio, and the like, each of which is optionally substituted. The term "thio derivative" also includes thiocarbamate, and the like.

As used herein, the term "acyl" includes formyl, and alkylcarbonyl, 25 alkenylcarbonyl, alkynylcarbonyl, heteroalkylcarbonyl, heteroalkenylcarbonyl, heteroalkynylcarbonyl, cycloalkylcarbonyl, cycloalkenylcarbonyl, cycloheteroalkylcarbonyl, cycloheteroalkenylcarbonyl, arylcarbonyl, arylalkylcarbonyl, arylalkenylcarbonyl, arylalkynylcarbonyl, heteroarylcarbonyl, heteroarylalkylcarbonyl, heteroarylalkenylcarbonyl, heteroarylalkynylcarbonyl, acylcarbonyl, and the like, each of which is optionally substituted.

30 As used herein, the term "carbonyl and derivatives thereof" includes the group C(O), C(S), C(NH) and substituted amino derivatives thereof.

As used herein, the term "carboxylic acid and derivatives thereof" includes the group CO₂H and salts thereof, and esters and amides thereof, and CN.

As used herein, the term "sulfinic acid or a derivative thereof" includes SO₂H 35 and salts thereof, and esters and amides thereof.

As used herein, the term "sulfonic acid or a derivative thereof" includes SO_3H and salts thereof, and esters and amides thereof.

As used herein, the term "sulfonyl" includes alkylsulfonyl, alkenylsulfonyl, alkynylsulfonyl, heteroalkylsulfonyl, heteroalkenylsulfonyl, heteroalkynylsulfonyl, cycloalkylsulfonyl, cycloalkenylsulfonyl, cycloheteroalkylsulfonyl, cycloheteroalkenylsulfonyl, arylsulfonyl, arylalkylsulfonyl, arylalkenylsulfonyl, arylalkynylsulfonyl, heteroarylsulfonyl, heteroarylalkylsulfonyl, heteroarylalkenylsulfonyl, heteroarylalkynylsulfonyl, acylsulfonyl, and the like, each of which is optionally substituted.

As used herein, the term "phosphinic acid or a derivative thereof" includes $\text{P(R)O}_2\text{H}$ and salts thereof, and esters and amides thereof, where R is alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, heteroalkyl, heteroalkenyl, cycloheteroalkyl, cycloheteroalkenyl, aryl, heteroaryl, arylalkyl, or heteroarylalkyl, each of which is optionally substituted.

As used herein, the term "phosphonic acid or a derivative thereof" includes PO_3H_2 and salts thereof, and esters and amides thereof.

As used herein, the term "hydroxylamino and derivatives thereof" includes NHOH , and alkyloxyNH, alkenyloxyNH, alkynyloxyNH, heteroalkyloxyNH, heteroalkenyloxyNH, heteroalkynyloxyNH, cycloalkyloxyNH, cycloalkenyloxyNH, cycloheteroalkyloxyNH, cycloheteroalkenyloxyNH, aryloxyNH, arylalkyloxyNH, arylalkenyloxyNH, arylalkynyloxyNH, heteroaryloxyNH, heteroarylalkyloxyNH, heteroarylalkenyloxyNH, heteroarylalkynyloxyNH, acyloxy, and the like, each of which is optionally substituted.

As used herein, the term "hydrazino and derivatives thereof" includes alkylNHNH, alkenylNHNH, alkynylNHNH, heteroalkylNHNH, heteroalkenylNHNH, heteroalkynylNHNH, cycloalkylNHNH, cycloalkenylNHNH, cycloheteroalkylNHNH, cycloheteroalkenylNHNH, arylNHNH, arylalkylNHNH, arylalkenylNHNH, arylalkynylNHNH, heteroarylNHNH, heteroarylalkylNHNH, heteroarylalkenylNHNH, heteroarylalkynylNHNH, acylNHNH, and the like, each of which is optionally substituted.

The term "optionally substituted" as used herein includes the replacement of hydrogen atoms with other functional groups on the radical that is optionally substituted. Such other functional groups illustratively include, but are not limited to, amino, hydroxyl, halo, thiol, alkyl, haloalkyl, heteroalkyl, aryl, arylalkyl, arylheteroalkyl, heteroaryl, heteroarylalkyl, heteroarylheteroalkyl, nitro, sulfonic acids and derivatives thereof, carboxylic acids and derivatives thereof, and the like. Illustratively, any of amino, hydroxyl, thiol, alkyl, haloalkyl, heteroalkyl, aryl, arylalkyl, arylheteroalkyl, heteroaryl, heteroarylalkyl, heteroarylheteroalkyl, and/or sulfonic acid is optionally substituted.

As used herein, the terms "optionally substituted aryl" and "optionally substituted heteroaryl" include the replacement of hydrogen atoms with other functional groups on the aryl or heteroaryl that is optionally substituted. Such other functional groups illustratively include, but are not limited to, amino, hydroxy, halo, thio, alkyl, haloalkyl, heteroalkyl, aryl, arylalkyl, arylheteroalkyl, heteroaryl, heteroarylalkyl, heteroarylheteroalkyl, nitro, sulfonic acids and derivatives thereof, carboxylic acids and derivatives thereof, and the like. Illustratively, any of amino, hydroxy, thio, alkyl, haloalkyl, heteroalkyl, aryl, arylalkyl, arylheteroalkyl, heteroaryl, heteroarylalkyl, heteroarylheteroalkyl, and/or sulfonic acid is optionally substituted.

Illustrative substituents include, but are not limited to, a radical $-(CH_2)_xZ^X$, where x is an integer from 0-6 and Z^X is selected from halogen, hydroxy, alkanoyloxy, including C₁-C₆ alkanoyloxy, optionally substituted aryloxy, alkyl, including C₁-C₆ alkyl, alkoxy, including C₁-C₆ alkoxy, cycloalkyl, including C₃-C₈ cycloalkyl, cycloalkoxy, including C₃-C₈ cycloalkoxy, alkenyl, including C₂-C₆ alkenyl, alkynyl, including C₂-C₆ alkynyl, haloalkyl, including C₁-C₆ haloalkyl, haloalkoxy, including C₁-C₆ haloalkoxy, halocycloalkyl, including C₃-C₈ halocycloalkyl, halocycloalkoxy, including C₃-C₈ halocycloalkoxy, amino, C₁-C₆ alkylamino, (C₁-C₆ alkyl)(C₁-C₆ alkyl)amino, alkylcarbonylamino, N-(C₁-C₆ alkyl)alkylcarbonylamino, aminoalkyl, C₁-C₆ alkylaminoalkyl, (C₁-C₆ alkyl)(C₁-C₆ alkyl)aminoalkyl, alkylcarbonylaminoalkyl, N-(C₁-C₆ alkyl)alkylcarbonylaminoalkyl, cyano, and nitro; or Z^X is selected from $-CO_2R^4$ and $-CONR^5R^6$, where R^4 , R^5 , and R^6 are each independently selected in each occurrence from hydrogen, C₁-C₆ alkyl, aryl-C₁-C₆ alkyl, and heteroaryl-C₁-C₆ alkyl.

The term "prodrug" as used herein generally refers to any compound that when administered to a biological system generates a biologically active compound as a result of one or more spontaneous chemical reaction(s), enzyme-catalyzed chemical reaction(s), and/or metabolic chemical reaction(s), or a combination thereof. In vivo, the prodrug is typically acted upon by an enzyme (such as esterases, amidases, phosphatases, and the like), simple biological chemistry, or other process in vivo to liberate or regenerate the more pharmacologically active drug. This activation may occur through the action of an endogenous host enzyme or a non-endogenous enzyme that is administered to the host preceding, following, or during administration of the prodrug. Additional details of prodrug use are described in U.S. Pat. No. 5,627,165; and Pathalk et al., Enzymic protecting group techniques in organic synthesis, Stereocel. Biocatal. 775-797 (2000). It is appreciated that the prodrug is advantageously converted to the original drug as soon as the goal, such as targeted delivery, safety, stability, and the like is achieved, followed by the subsequent rapid elimination of the released remains

of the group forming the prodrug.

Prodrugs may be prepared from the compounds described herein by attaching groups that ultimately cleave in vivo to one or more functional groups present on the compound, such as -OH-, -SH-, -CO₂H-, -NR₂. Illustrative prodrugs include but are not limited to carboxylate esters where the group is alkyl, aryl, arylalkyl, heteroaryl, heteroarylalkyl, acyloxyalkyl, alkoxycarbonyloxyalkyl as well as esters of hydroxyl, thiol and amines where the group attached is an acyl group, an alkoxycarbonyl, aminocarbonyl, phosphate or sulfate. Illustrative esters, also referred to as active esters, include but are not limited to 1-indanyl, N-oxysuccinimide; acyloxyalkyl groups such as acetoxymethyl, pivaloyloxymethyl, β-acetoxylethyl, β-pivaloyloxyethyl, 1-(cyclohexylcarbonyloxy)prop-1-yl, (1-aminoethyl)carbonyloxymethyl, and the like; alkoxycarbonyloxyalkyl groups, such as ethoxycarbonyloxymethyl, α-ethoxycarbonyloxyethyl, β-ethoxycarbonyloxyethyl, and the like; dialkylaminoalkyl groups, including di-lower alkylamino alkyl groups, such as dimethylaminomethyl, dimethylaminoethyl, diethylaminomethyl, diethylaminoethyl, and the like; 2-(alkoxycarbonyl)-2-alkenyl groups such as 2-(isobutoxycarbonyl) pent-2-enyl, 2-(ethoxycarbonyl)but-2-enyl, and the like; and lactone groups such as phthalidyl, dimethoxyphthalidyl, and the like.

Further illustrative prodrugs contain a chemical moiety, such as an amide or phosphorus group functioning to increase solubility and/or stability of the compounds described herein. Further illustrative prodrugs for amino groups include, but are not limited to, (C₃-C₂₀)alkanoyl; halo-(C₃-C₂₀)alkanoyl; (C₃-C₂₀)alkenoyl; (C₄-C₇)cycloalkanoyl; (C₃-C₆)-cycloalkyl(C₂-C₁₆)alkanoyl; optionally substituted aroyl, such as unsubstituted aroyl or aroyl substituted by 1 to 3 substituents selected from the group consisting of halogen, cyano, trifluoromethanesulphonyloxy, (C₁-C₃)alkyl and (C₁-C₃)alkoxy, each of which is optionally further substituted with one or more of 1 to 3 halogen atoms; optionally substituted aryl(C₂-C₁₆)alkanoyl and optionally substituted heteroaryl(C₂-C₁₆)alkanoyl, such as the aryl or heteroaryl radical being unsubstituted or substituted by 1 to 3 substituents selected from the group consisting of halogen, (C₁-C₃)alkyl and (C₁-C₃)alkoxy, each of which is optionally further substituted with 1 to 3 halogen atoms; and optionally substituted heteroarylalkanoyl having one to three heteroatoms selected from O, S and N in the heteroaryl moiety and 2 to 10 carbon atoms in the alkanoyl moiety, such as the heteroaryl radical being unsubstituted or substituted by 1 to 3 substituents selected from the group consisting of halogen, cyano, trifluoromethanesulphonyloxy, (C₁-C₃)alkyl, and (C₁-C₃)alkoxy, each of which is optionally further substituted with 1 to 3 halogen atoms. The groups illustrated are exemplary, not exhaustive, and may be prepared by conventional processes.

It is understood that the prodrugs themselves may not possess significant biological activity, but instead undergo one or more spontaneous chemical reaction(s), enzyme-catalyzed chemical reaction(s), and/or metabolic chemical reaction(s), or a combination thereof after administration in vivo to produce the compound described herein that is biologically active or is a precursor of the biologically active compound. However, it is appreciated that in some cases, the prodrug is biologically active. It is also appreciated that prodrugs may often serve to improve drug efficacy or safety through improved oral bioavailability, pharmacodynamic half-life, and the like. Prodrugs also refer to derivatives of the compounds described herein that include groups that simply mask undesirable drug properties or improve drug delivery. For example, one or more compounds described herein may exhibit an undesirable property that is advantageously blocked or minimized may become pharmacological, pharmaceutical, or pharmacokinetic barriers in clinical drug application, such as low oral drug absorption, lack of site specificity, chemical instability, toxicity, and poor patient acceptance (bad taste, odor, pain at injection site, and the like), and others. It is appreciated herein that a prodrug, or other strategy using reversible derivatives, can be useful in the optimization of the clinical application of a drug.

As used herein, the term "composition" generally refers to any product comprising the specified ingredients in the specified amounts, as well as any product which results, directly or indirectly, from combinations of the specified ingredients in the specified amounts. It is to be understood that the compositions described herein may be prepared from isolated compounds described herein or from salts, solutions, hydrates, solvates, and other forms of the compounds described herein. It is also to be understood that the compositions may be prepared from various amorphous, non-amorphous, partially crystalline, crystalline, and/or other morphological forms of the compounds described herein. It is also to be understood that the compositions may be prepared from various hydrates and/or solvates of the compounds described herein. Accordingly, such pharmaceutical compositions that recite compounds described herein are to be understood to include each of, or any combination of, the various morphological forms and/or solvate or hydrate forms of the compounds described herein. Illustratively, compositions may include one or more carriers, diluents, and/or excipients. The compounds described herein, or compositions containing them, may be formulated in a therapeutically effective amount in any conventional dosage forms appropriate for the methods described herein. The compounds described herein, or compositions containing them, including such formulations, may be administered by a wide variety of conventional routes for the methods described herein, and in a wide variety of dosage formats, utilizing known procedures (see generally, Remington: The Science and Practice of Pharmacy, (21st ed., 2005)).

The term "therapeutically effective amount" as used herein, refers to that amount of active compound or pharmaceutical agent that elicits the biological or medicinal response in a tissue system, animal or human that is being sought by a researcher, veterinarian, medical doctor or other clinician, which includes alleviation of the symptoms of the disease or disorder being treated. In one aspect, the therapeutically effective amount is that which may treat or alleviate the disease or symptoms of the disease at a reasonable benefit/risk ratio applicable to any medical treatment. However, it is to be understood that the total daily usage of the compounds and compositions described herein may be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically-effective dose level for any particular patient will depend upon a variety of factors, including the disorder being treated and the severity of the disorder; activity of the specific compound employed; the specific composition employed; the age, body weight, general health, gender and diet of the patient; the time of administration, route of administration, and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidentally with the specific compound employed; and like factors well known to the researcher, veterinarian, medical doctor or other clinician of ordinary skill.

It is also appreciated that the therapeutically effective amount, whether referring to monotherapy or combination therapy, is advantageously selected with reference to any toxicity, or other undesirable side effect, that might occur during administration of one or more of the compounds described herein. Further, it is appreciated that the co-therapies described herein may allow for the administration of lower doses of compounds that show such toxicity, or other undesirable side effect, where those lower doses are below thresholds of toxicity or lower in the therapeutic window than would otherwise be administered in the absence of a cotherapy.

In addition to the illustrative dosages and dosing protocols described herein, it is to be understood that an effective amount of any one or a mixture of the compounds described herein can be readily determined by the attending diagnostician or physician by the use of known techniques and/or by observing results obtained under analogous circumstances. In determining the effective amount or dose, a number of factors are considered by the attending diagnostician or physician, including, but not limited to the species of mammal, including human, its size, age, and general health, the specific disease or disorder involved, the degree of or involvement or the severity of the disease or disorder, the response of the individual patient, the particular compound administered, the mode of administration, the bioavailability characteristics of the preparation administered, the dose regimen selected, the use of concomitant medication, and other relevant circumstances.

The dosage of each compound of the claimed combinations depends on several factors, including: the administration method, the condition to be treated, the severity of the condition, whether the condition is to be treated or prevented, and the age, weight, and health of the person to be treated. Additionally, pharmacogenomic (the effect of genotype on the pharmacokinetic, pharmacodynamic or efficacy profile of a therapeutic) information about a particular patient may affect the dosage used.

It is to be understood that in the methods described herein, the individual components of a co-administration, or combination can be administered by any suitable means, contemporaneously, simultaneously, sequentially, separately or in a single pharmaceutical formulation. Where the co-administered compounds or compositions are administered in separate dosage forms, the number of dosages administered per day for each compound may be the same or different. The compounds or compositions may be administered via the same or different routes of administration. The compounds or compositions may be administered according to simultaneous or alternating regimens, at the same or different times during the course of the therapy, concurrently in divided or single forms.

The term "administering" as used herein includes all means of introducing the compounds and compositions described herein to the patient, including, but are not limited to, oral (po), intravenous (iv), intramuscular (im), subcutaneous (sc), transdermal, inhalation, buccal, ocular, sublingual, vaginal, rectal, and the like. The compounds and compositions described herein may be administered in unit dosage forms and/or formulations containing conventional nontoxic pharmaceutically-acceptable carriers, adjuvants, and/or vehicles.

Illustrative formats for oral administration include tablets, capsules, elixirs, syrups, and the like.

Illustrative routes for parenteral administration include intravenous, intraarterial, intraperitoneal, epidural, intraurethral, intrasternal, intramuscular and subcutaneous, as well as any other art recognized route of parenteral administration.

Illustratively, administering includes local use, such as when administered locally to the site of disease, injury, or defect, or to a particular organ or tissue system. Illustrative local administration may be performed during open surgery, or other procedures when the site of disease, injury, or defect is accessible. Alternatively, local administration may be performed using parenteral delivery where the compound or compositions described herein are deposited locally to the site without general distribution to multiple other non-target sites in the patient being treated. It is further appreciated that local administration may be directly in the injury site, or locally in the surrounding tissue. Similar variations regarding local delivery to particular tissue types, such as organs, and the like, are also described herein. Illustratively,

compounds may be administered directly to the nervous system including, but not limited to, intracerebral, intraventricular, intracerebroventricular, intrathecal, intracisternal, intraspinal and/or peri-spinal routes of administration by delivery via intracranial or intravertebral needles and/or catheters with or without pump devices.

5 Depending upon the disease as described herein, the route of administration and/or whether the compounds and/or compositions are administered locally or systemically, a wide range of permissible dosages are contemplated herein, including doses falling in the range from about 1 µg/kg to about 1 g/kg. The dosages may be single or divided, and may administered according to a wide variety of protocols, including q.d., b.i.d., t.i.d., or even every
10 other day, once a week, once a month, once a quarter, and the like. In each of these cases it is understood that the therapeutically effective amounts described herein correspond to the instance of administration, or alternatively to the total daily, weekly, month, or quarterly dose, as determined by the dosing protocol.

 In making the pharmaceutical compositions of the compounds described herein,
15 a therapeutically effective amount of one or more compounds in any of the various forms described herein may be mixed with one or more excipients, diluted by one or more excipients, or enclosed within such a carrier which can be in the form of a capsule, sachet, paper, or other container. Excipients may serve as a diluent, and can be solid, semi-solid, or liquid materials, which act as a vehicle, carrier or medium for the active ingredient. Thus, the formulation
20 compositions can be in the form of tablets, pills, powders, lozenges, sachets, cachets, elixirs, suspensions, emulsions, solutions, syrups, aerosols (as a solid or in a liquid medium), ointments, soft and hard gelatin capsules, suppositories, sterile injectable solutions, and sterile packaged powders. The compositions may contain anywhere from about 0.1% to about 99.9% active ingredients, depending upon the selected dose and dosage form.

25 The following examples further illustrate specific embodiments of the invention; however, the following illustrative examples should not be interpreted in any way to limit the invention.

EXAMPLES

 METHOD EXAMPLE. Cell culture. MC3T3 E1 mouse osteoblast-like cells
30 (clone 14 – MC3T3 E1-14; and no clonal cells in supplementary figures), and RAW264.7 mouse pre-osteoclast (monocyte/macrophage) cells were cultured in αMEM containing 10% fetal bovine serum and antibiotics (50 units/ml penicillin, and 50 µg/ml streptomycin; Life Technologies, Grand Island, NY, USA). Cells were maintained at 37°C and 5% CO₂ in a humidified incubator. Cell mortality and live cell numbers were determined 24 h after the
35 treatment with 20 ng/ml RANKL (PeproTech, Rocky Hills, NC, USA) in response to 0.1 – 20

μM salubrinal or 1 – 20 μM guanabenz acetate (Tocris Bioscience, Ellisville, MO, USA). Cells were stained with trypan blue and the numbers of live and dead cells were counted using a hemacytometer.

METHOD EXAMPLE. Inhibition of osteoclastogenesis of RAW264.7 cells by salubrinal. The primary aim of this study is to evaluate the effects of salubrinal on osteoclastogenesis. In response to 0.1 – 20 μM salubrinal for 24 h, cell mortality and live cell numbers of RAW264.7 pre-osteoclasts was examined. Cell mortality ratio did not present statistically significant differences in the presence and absence of RANKL (Fig. 3A). The number of live cells was increased by ~50% by incubation with RANKL, and administration of 10 - 20 μM salubrinal reduced the numbers approximately by 10% (Fig. 3B). Consistent with the stimulatory role of RANKL, the number of TRAP-positive multi-nucleated cells was substantially increased by addition of RANKL. However, administration of 0.5 μM to 20 μM salubrinal reduced the number of TRAP-positive cells in a dose dependent manner (Fig. 3C & 3D).

METHOD EXAMPLE. Inhibitory effects of guanabenz on osteoclastogenesis of RAW264.7 cells. To further examine a potential involvement of p-eIF2 α in regulation of osteoclastogenesis, we employed guanabenz that also acts as an inhibitor of de-phosphorylation of eIF2 α was evaluated. Administration of 1 and 5 μM guanabenz did not alter cell mortality and the number of live cells, although its administration at 10 and 20 μM reduced the number of live cells in 24 h (Fig. 7A & 7B). Consistent with salubrinal's inhibitory action, guanabenz also attenuated osteoclastogenesis of RAW264.7 cells in a dose dependent manner (Fig. 7C & 7D). Compared to the number of TRAP-positive multi-nucleated cells of 377 ± 39 (RANKL only), guanabenz reduced the number of differentiated osteoclasts to 364 ± 38 (1 μM), 288 ± 51 (5 μM), 189 ± 25 (10 μM), and 73 ± 16 (20 μM).

METHOD EXAMPLE. Quantitative real-time PCR. Total RNA was extracted using an RNeasy Plus mini kit (Qiagen, Germantown, MD, USA). Reverse transcription was conducted with high capacity cDNA reverse transcription kits (Applied Biosystems, Carlsbad, CA, USA), and quantitative real-time PCR was performed using ABI 7500 with Power SYBR green PCR master mix kits (Applied Biosystems). We evaluated mRNA levels of ATF4, Osteocalcin (OCN), NFATc1, c-Fos, tartrate-resistant acid phosphatase (TRAP), and osteoclast-associated receptor (OSCAR) with the PCR primers listed in Table 1. GAPDH was used for internal control. The relative mRNA abundance for the selected genes with respect to the level of GAPDH mRNA was expressed as a ratio of $S_{\text{treated}}/S_{\text{control}}$, where S_{treated} = mRNA level for the cells treated with chemical agents, and S_{control} = mRNA level for control cells [23].

Table 1. Real-time PCR primers used in this study.

target	forward primer	backward primer
ATF4	5'- TGGCGAGTGTAAGGAGCTAGAAA -3'	5'- TCTTCCCCCTTGCCTTACG -3'
OCN	5'- CCGGGAGCAGTGTGAGCTTA -3'	5'- AGGCGGTCTTCAAGCCATACT -3'
NFATc1	5'- GGTGCTGTCTGGCCATAACT -3'	5'- GCGGAAAGGTGGTATCTCAA -3'
c-Fos	5'- AGGCCAGTGGCTCAGAGA -3'	5'- CCAGTCTGCTGCATAGAAGGAA -3'
TRAP	5'- TCCTGGCTCAAAAAGCAGTT -3'	5'- ACATAGCCCACACCGTTCTC -3'
OSCAR	5'- ACACACACACCTGGCACCTA -3'	5'- GAGACCATCAAAGGCAGAGC -3'
GAPDH	5'- TGCACCACCAACTGCTTAG -3'	5'- GGATGCAGGGATGATGTTTC -3'

METHOD EXAMPLE. Downregulation of NFATc1 in RAW264.7 cells by salubrinal. NFATc1 is a transcription factor critical for activating osteoclastogenesis. Addition of RANKL to the culture medium significantly induced NFATc1 expression day 2 and maintained its elevated level on day 4 (Fig. 4). The RANKL-induced expression of NFATc1 was reduced by administration of 5 – 20 μ M salubrinal on both days, and the effect of salubrinal was dose dependent (Fig. 4).

METHOD EXAMPLE. Partial suppression of mRNA levels of NFATc1, c-Fos, TRAP, and OSCAR by salubrinal. Addition of RANKL increased the mRNA levels of NFATc1, c-Fos, TRAP, and OSCAR, and administration of 20 μ M salubrinal significantly reduced their mRNA levels. On day 2, for instance, the RANKL-driven increase was 9.4 ± 0.5 fold (NFATc1), 1.9 ± 0.1 fold (c-fos), 165 ± 4.2 fold (TRAP), and 467 ± 22 fold (OSCAR). The reduction by 20 μ M salubrinal was 46% (NFATc1), 32% (c-fos), 35% (TRAP), and 21% (OSCAR) (Fig. 5A). Consistent with the observed dose response, administration of salubrinal at 0.1 - 1 μ M did not contribute to significant reduction in these mRNA levels except for NFATc1 and c-fos on day 4 (Fig. 5B).

METHOD EXAMPLE. Reduction of RANKL-induced NFATc1, c-Fos, TRAP, and OSCAR by guanabenz. The induction of NFATc1 by RANKL was suppressed by guanabenz in a dose dependent manner (Fig. 8A). The mRNA levels of NFATc1, c-Fos, TRAP, and OSCAR were also reduced by administration of 20 μ M guanabenz. Lower concentrations of guanabenz, 5 and 10 μ M, were effective in reducing the levels of TRAP and OSCAR mRNA (Fig. 8B). The temporal expression profile of p-eIF2 α and NFATc1 in response to 20 μ M guanabenz revealed that p-eIF2 α was upregulated in 2 h and NFATc1 was partially suppressed in 8 h (Fig. 9). The normalized level of “1” was defined as the level for the cells that were not treated with RANKL without administration of guanabenz. In the absence of RANKL administration, however, either salubrinal or guanabenz did not significantly alter cell mortality and expression of NFATc1 and TRAP (Supplementary Fig. S4).

METHOD EXAMPLE. Western Immunoblotting. Cells were lysed in a

radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phosphatase inhibitors (Calbiochem, Billerica, MA, USA). Isolated proteins were fractionated using 10-15% SDS gels and electro-transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The membrane was incubated for 1 h with primary antibodies followed by 45 min incubation with goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase (Cell Signaling, Danvers, MA, USA). We used antibodies against ATF4, NFATc1 (Santa Cruz), p-eIF2 α (Thermo Scientific, Waltham, MA, USA), eIF2 α , caspase 3, cleaved caspase 3, p38 and p-p38 mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and p-ERK, nuclear factor kappa B (NF κ B) p65 and p-NF κ B p65 (Cell Signaling), and β -actin (Sigma). Protein levels were assayed using a SuperSignal west femto maximum sensitivity substrate (Thermo Scientific), and signal intensities were quantified with a luminescent image analyzer (LAS-3000, Fuji Film, Tokyo, Japan).

METHOD EXAMPLE. Temporal profile of p-eIF2 α and NFATc1. The temporal expression profile revealed that addition of RANKL transiently reduced the phosphorylation level of eIF2 α (2 - 8 h) and elevated NFATc1 by 13.4 ± 3.2 fold (24 h) (Fig. 6). This induction of NFATc1 was partially suppressed by salubrinal with an increase in the level of p-eIF2 α . In the early period (2 - 4 h), administration of 20 μ M salubrinal increased the level of p-eIF2 α but did not alter the level of NFATc1. In the later period (8 - 24 h), however, the level of NFATc1 was significantly reduced by 48% (8 h) and 44% (24 h). Administration of 20 μ M salubrinal did not significantly alter the phosphorylation level of ERK, p38 MAPK, and NF κ B (Fig. 6). Note that the normalized level of "1" in Fig. 6C was defined as the level for the cells that were not treated with RANKL without administration of guanabenz.

METHOD EXAMPLE. Knockdown of ATF4 and eIF2 α by siRNA. Cells were treated with siRNA specific to ATF4 and eIF2 α (Life Technologies). Selected target sequences for knockdown of ATF4 and eIF2 α were: ATF4, 5'-GCU GCU UAC AUU ACU CUA A-3'; and eIF2 α , 5'-CGG UCA AAA UUC GAG CAG A-3'. As a nonspecific control, a negative siRNA (Silencer Select #1, Life Technologies) was used. Cells were transiently transfected with siRNA for ATF4, eIF2 α or control in Opti-MEM I medium with Lipofectamine RNAiMAX (Life Technologies). Six hours later, the medium was replaced by regular culture medium. The efficiency of silencing was assessed with immunoblotting or quantitative PCR 48 h after transfection.

METHOD EXAMPLE. ATF4-mediated elevation of osteocalcin mRNA in MC3T3 E1-14 cells. Salubrinal is an inhibitor of de-phosphorylation of eIF2 α . Administration

of 5 μ M salubrinal to MC3T3 E1-14 cells elevated phosphorylation of eIF2 α , followed by an increase in ATF4 expression (Fig. 2A). Furthermore, the level of osteocalcin mRNA was increased 3.3 ± 0.5 fold (24 h) and 3.3 ± 0.3 fold (32 h) (Fig. 2B). When expression of ATF4 was significantly reduced by RNA interference (Fig. 2C & 2D), however, salubrinal-driven elevation of the osteocalcin mRNA level was suppressed (Fig. 2E). Non-clonal MC3T3 E1 cells also presented elevation of p-eIF2 α and ATF4, together with an increase in the mRNA levels of ATF4 and osteocalcin (Supplementary Fig. S2). In addition, administration of guanabenz to MC3T3 E1-14 elevated the mRNA level of osteocalcin in a dose dependent manner, consistent with an increase in p-eIF2 α and ATF4 (Supplementary Fig. S3).

METHOD EXAMPLE. Reduction in salubrinal/guanabenz-driven suppression of NFATc1 expression by RNA interference for eIF2 α . To evaluate the effects of eIF2 α on the expression level of NFATc1, RNA interference specific for eIF2 α together with a non-specific control (NC) was used (Fig. 10). In response to 20 μ M salubrinal, RAW264.7 cells transfected with the control siRNA demonstrated a reduction of NFATc1 by 56%. However, the expression of NFATc1 was reduced only by 20% in the cells transfected with eIF2 α siRNA. Furthermore, 20 μ M guanabenz decreased the level of NFATc1 by 43% in the cells transfected with the control siRNA but the transfection of eIF2 α siRNA abolished the suppressive effect of guanabenz. The phosphorylation level of NF κ B was not significantly altered by transfection with eIF2 α siRNA.

METHOD EXAMPLE. Mineralization assay. Mineralization of extracellular matrix was assayed by Alizarin Red S staining. MC3T3-E1 cells were plated in 6-well plates. When cells were confluent, 50 μ g/ml of ascorbic acid (Wako Chemicals, Richmond, VA, USA) and 5 mM β -glycerophosphate (Sigma) were added. The medium was changed every other day, and staining was conducted after 3 week. Cells were washed with PBS twice and fixed with 60% isopropanol for 1 min at room temperature, followed by rehydration with distilled water for 3 min at room temperature. They were stained with 1% Alizarin red S (Sigma) for 3 min and washed with distilled water.

METHOD EXAMPLE. Enhanced mineralization of MC3T3 E1-14 cells by salubrinal. Prior to examining the effects of salubrinal on osteoclastogenesis, its effects on development of osteoblasts was tested, focusing on cell viability, phosphorylation of eIF2 α (p-eIF2 α), expression of ATF4 and osteocalcin, and matrix mineralization. Administration of 5 – 20 μ M salubrinal to MC3T3 E1-14 cells did not increase cell mortality or inhibit cell proliferation (Fig. 1A). Unlike application of 10 nM thapsigargin, which is a stress inducer to the endoplasmic reticulum that elevates p-eIF2 α , incubation with 10 μ M salubrinal for 24 h did

not elevate the expression level of cleaved caspase 3 (Fig. 1B). After 3-week incubation in an osteogenic medium, Alizarin red S staining area showed that salubrinal enhanced mineralization of MC3T3 E1-14 cells in a dose dependent manner (Fig. 1C). The enhanced mineralization was also observed in non-clonal MC3T3 E1 cells (Supplementary Fig. S1).

5 METHOD EXAMPLE. Osteoclastogenesis in vitro and TRAP (Tartrate-resistant acid phosphatase) staining. RAW264.7 cells were plated at a density of $5 \times 10^3/\text{cm}^2$ into a 12-well or a 60 mm dish, and cultured with 20 ng/ml RANKL in the presence and absence of salubrinal or guanabenz. The culture medium was replaced every 2 days. After 5 days of culture, the cells were stained for TRAP staining using an acid phosphatase leukocyte
10 kit (Sigma). The number of TRAP-positive cells containing three or more nuclei was determined.

 METHOD EXAMPLE. Statistical analysis. Three or four-independent experiments were conducted and data were expressed as mean \pm S.D. For comparison among multiple samples, ANOVA followed by post hoc tests was conducted. Statistical significance
15 was evaluated at $p < 0.05$. The single and double asterisks and daggers indicate $p < 0.05$ and $p < 0.01$. To determine intensities in immunoblotting and areas of Alizarin red S staining, images were scanned with Adobe Photoshop CS2 (Adobe Systems, San Jose, CA, USA) and quantified using Image J.

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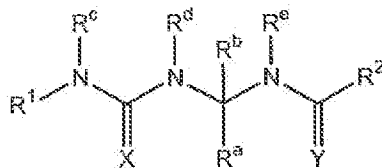
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WHAT IS CLAIMED IS:

1. A method for treating osteoporosis, fracture, or a bone defect in a host animal, the method comprising the step of administering to the host animal a therapeutically effective amount of one or more compounds capable of selectively inhibiting dephosphorylation of eIF2 α ; where the amount is capable of (a) stimulating matrix deposition of osteoblasts, (b) upregulating activating transcription factor 4 (ATF4), (c) reducing expression of nuclear factor of activated T cells c1 (NFATc1), (d) inhibiting differentiation of RAW264.7 cells to multi-nucleated osteoclasts, (e) stimulating osteoblastogenesis, or (f) inhibiting osteoclastogenesis, or a combination of the foregoing, or a pharmaceutically acceptable composition comprising the one or more compounds.

2. The method claim 1 wherein at least one compound is of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X is O or S;

Y is O or S;

R¹ is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, or heteroaryl, each of which is optionally substituted;

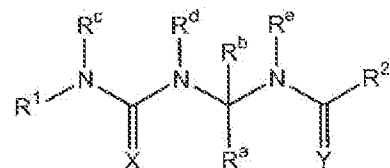
R² is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted;

R³ is optionally substituted alkyl;

R⁴ is H or optionally substituted C₁-C₆ alkyl;

R⁵, R⁶, and R⁷ are each independently selected from the group consisting of H, optionally substituted C₁-C₆ alkyl, acyl, or a prodrug capable of releasing the attached nitrogen in vivo.

3. The method claim 1 or 2 wherein at least one compound is of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X is O or S;

Y is 0 or S:

R¹ is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, or heteroaryl, each of which is optionally substituted;

R² is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted;

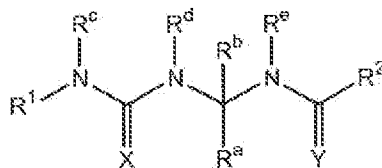
R² is optionally substituted alkyl;

R^b is H or optionally substituted C₁-C₆ alkyl;

R^c, R^d, and R^e are each independently selected from the group consisting of H, optionally substituted C₁-C₆ alkyl, acyl, or a prodrug capable of releasing the attached nitrogen in vivo;

or a composition thereof further comprising one or more carriers, diluents, or excipients, or a combination thereof.

4. The method of any one of the preceding claims wherein at least one compound is of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X is O or S;

Y is 0 or S;

R¹ is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, or heteroaryl, each of which is optionally substituted;

R² is alkyl, cycloalkyl, alkenyl, cycloalkenyl, aryl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted;

R^a is optionally substituted alkyl;

R^b is H or optionally substituted C₁-C₆ alkyl;

R^c, R^d, and R^e are each independently selected from the group consisting of H, optionally substituted C₁-C₆ alkyl, acyl, or a prodrug capable of releasing the attached nitrogen in vivo.

5. The method of any one of the preceding claims wherein R¹ is alkyl, aryl, or heteroaryl, each of which is optionally substituted.

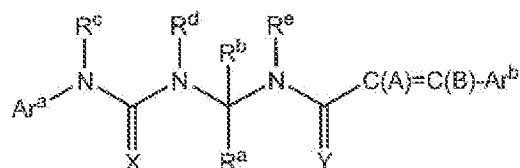
6. The method of any one of the preceding claims wherein R¹ is aryl or

heteroaryl, each of which is optionally substituted.

7. The method of any one of the preceding claims wherein R^2 is alkenyl, heteroaryl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted.

8. The method of any one of the preceding claims wherein R^2 is alkenyl, cycloalkylalkyl, cycloalkenylalkyl, arylalkyl, heteroarylalkyl, cycloalkylalkenyl, cycloalkenylalkenyl, arylalkenyl, or heteroarylalkenyl, each of which is optionally substituted.

9. The method of any one of the preceding claims wherein at least one compound is a compound of the formula



or a pharmaceutically acceptable salt thereof, wherein:

X and Y are independently O or S;

Ar^a and Ar^b are independently aryl or heteroaryl, each of which is optionally substituted;

R^a is optionally substituted alkyl;

R^b is H or optionally substituted C_1 - C_6 alkyl;

R^c , R^d , and R^e are each independently selected from the group consisting of H, optionally substituted C_1 - C_6 alkyl, acyl, and a prodrug capable of releasing the attached nitrogen in vivo to form the corresponding H or salt derivative thereof;

and A and B are independently H, or optionally substituted C_1 - C_6 alkyl

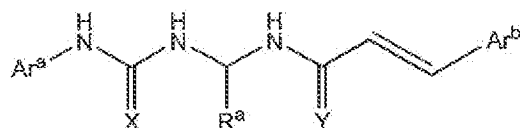
10. The method of claim 9 wherein the aryl is a bicyclic aryl.

11. The method of claim 9 or 10 wherein the heteroaryl is a bicyclic heteroaryl.

12. The method of any one of claims 9 to 11 wherein the aryl or heteroaryl is optionally substituted with one or more substituents selected from the group consisting of halo, nitrile, or optionally substituted C_1 - C_6 alkyl, C_1 - C_6 alkoxy, C_1 - C_6 haloalkyl, C_1 - C_6 haloalkoxy, aryl, aryloxy, heterocyclyl, heterocyclyloxy, fused aryl, and fused heterocyclyl.

13. The method of any one of claims 9 to 12 wherein the alkenyl has an E geometry.

14. The method of any one of the preceding claims wherein at least one compound is of the formula



or a pharmaceutically acceptable salt thereof, wherein,

X is O or S;

Y is O or S;

Ar^a and Ar^b are independently aryl or heteroaryl, each of which is optionally substituted; and

R^a is optionally substituted alkyl.

15. The method of claim 14 wherein the aryl is a bicyclic aryl.

16. The method of claim 14 or 15 wherein Ar^a is a bicyclic heteroaryl.

17. The method of any one of claims 14 to 16 wherein the aryl or heteroaryl is optionally substituted with one or more substituents selected from the group consisting of halo, nitrile, or optionally substituted C₁-C₆ alkyl, C₁-C₆ alkoxy, C₁-C₆ haloalkyl, C₁-C₆ haloalkoxy, aryl, aryloxy, heterocyclyl, heterocyclyloxy, fused aryl, and fused heterocyclyl.

18. The method of any one of claims 14 to 17 wherein R^a is haloalkyl.

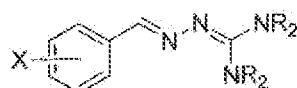
19. The method of any one of claims 14 to 18 wherein R^a is not trifluoromethyl.

20. The method of any one of claims 14 to 19 wherein R^a is haloalkyl, where halo is selected from the group consisting of chloro and bromo, and combinations thereof.

21. The method of any one of claims 14 to 20 wherein R^c, R^d, and R^e are each independently selected from the group consisting of H, optionally substituted C₁-C₆ alkyl, and acyl.

22. The method of any one of the preceding claims wherein at least one compound is salubrinol, or an analog or a derivative thereof, or a pharmaceutically acceptable salt of the foregoing.

23. The method of any one of the preceding claims wherein at least one compound is of the formula



or a pharmaceutically acceptable salt thereof, wherein

X represents five substituents each independently selected from hydrogen, halo, hydroxy and derivatives thereof, amino and derivatives thereof, thio and derivatives thereof, carboxylate or a derivative thereof, sulfinyl or a derivative thereof, sulfonyl or a derivative thereof, phosphinyl or a derivative thereof, or phosphonyl or a derivative thereof, or alkyl,

alkenyl, alkynyl, cycloalkyl, cycloalkenyl, heteroalkyl, heteroalkenyl, cycloheteroalkyl, cycloheteroalkenyl, aryl, heteroaryl, arylalkyl, or heteroarylalkyl, each of which is optionally substituted; and

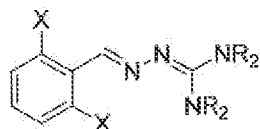
R is independently selected in each instance from hydrogen, hydroxy and derivatives thereof, amino and derivatives thereof, carboxylate and derivatives thereof, sulfonyl and derivatives thereof, and alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, heteroalkyl, heteroalkenyl, cycloheteroalkyl, cycloheteroalkenyl, aryl, heteroaryl, arylalkyl, and heteroarylalkyl, each of which is optionally substituted, and nitrogen prodrug forming groups.

24. The method of claim 23 wherein at least one X is halo, such as chloro.

25. The method of claim 23 wherein at least one X is optionally substituted alkyl, such as methyl or trifluoromethyl.

26. The method of claim 23 wherein at least one X is optionally substituted alkoxy, such as methoxy or trifluoromethoxy.

27. The method of any one of the preceding claims wherein at least one compound is of the formula



or a pharmaceutically acceptable salt thereof

28. The method of claim 27 wherein each X is halo.

29. The method of claim 27 wherein each X is chloro.

30. The method of any one of claims 27 to 29 wherein each R is hydrogen

31. The method of any one of claims 27 to 30 wherein at least one compound is guanabenz or a pharmaceutically acceptable salt thereof.

32. The method of any one of the preceding claims wherein the composition further comprises one or more carriers, diluents, or excipients, or a combination thereof.

33. The method of any one of the preceding claims wherein the host animal is a human.

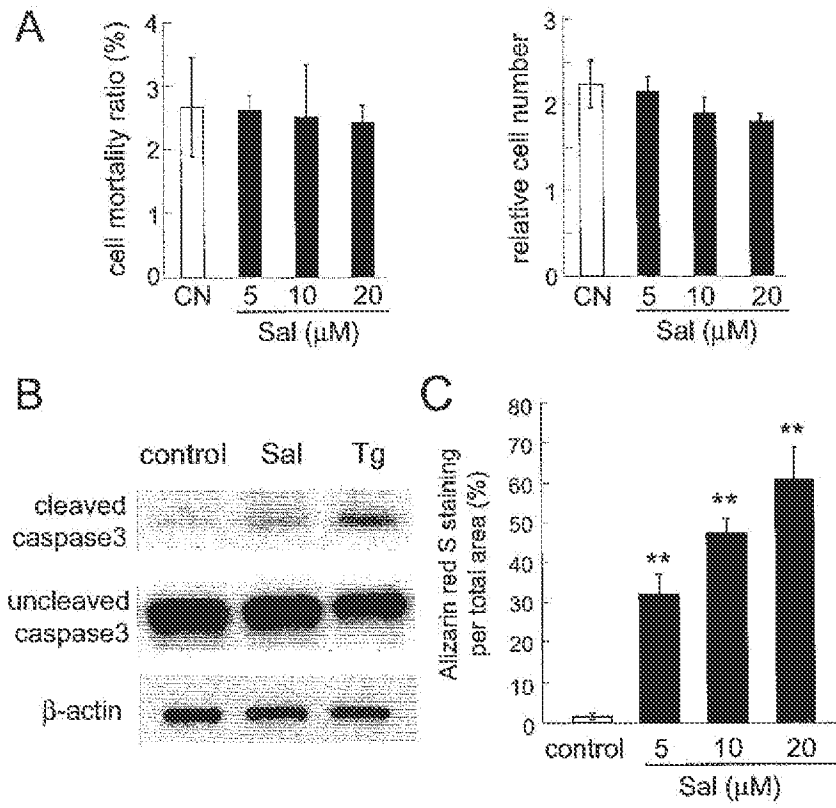


Figure 1

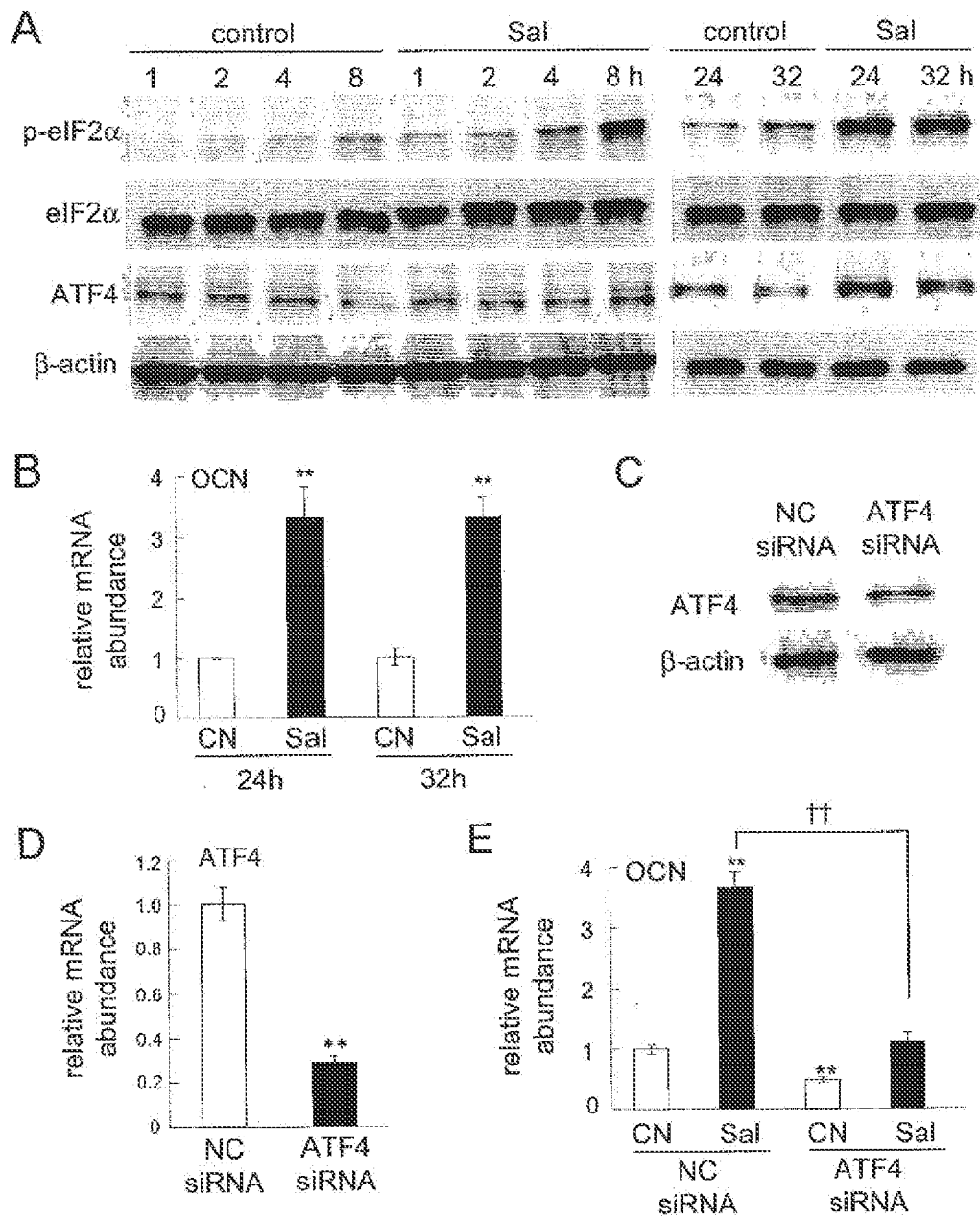


Figure 2

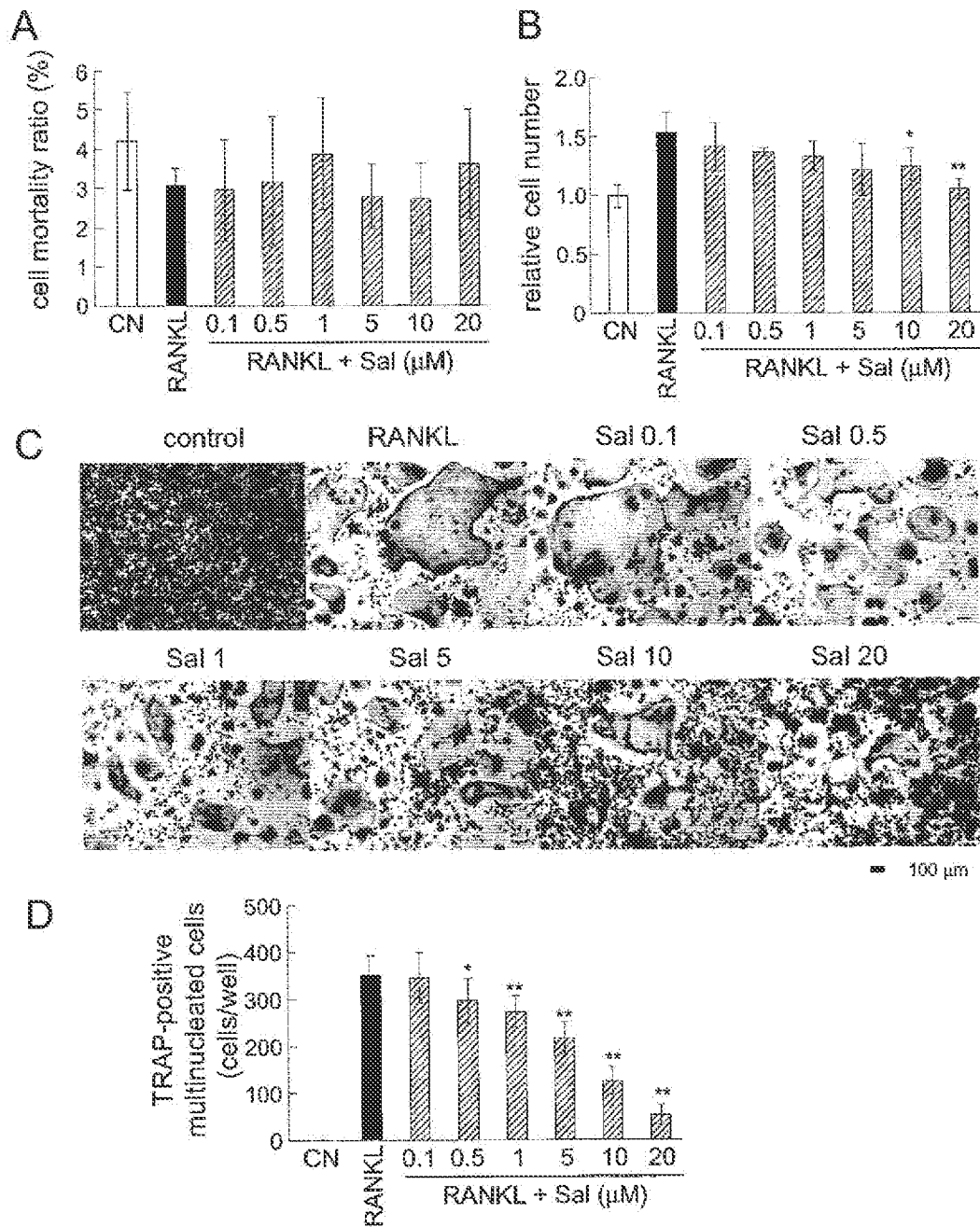


Figure 3

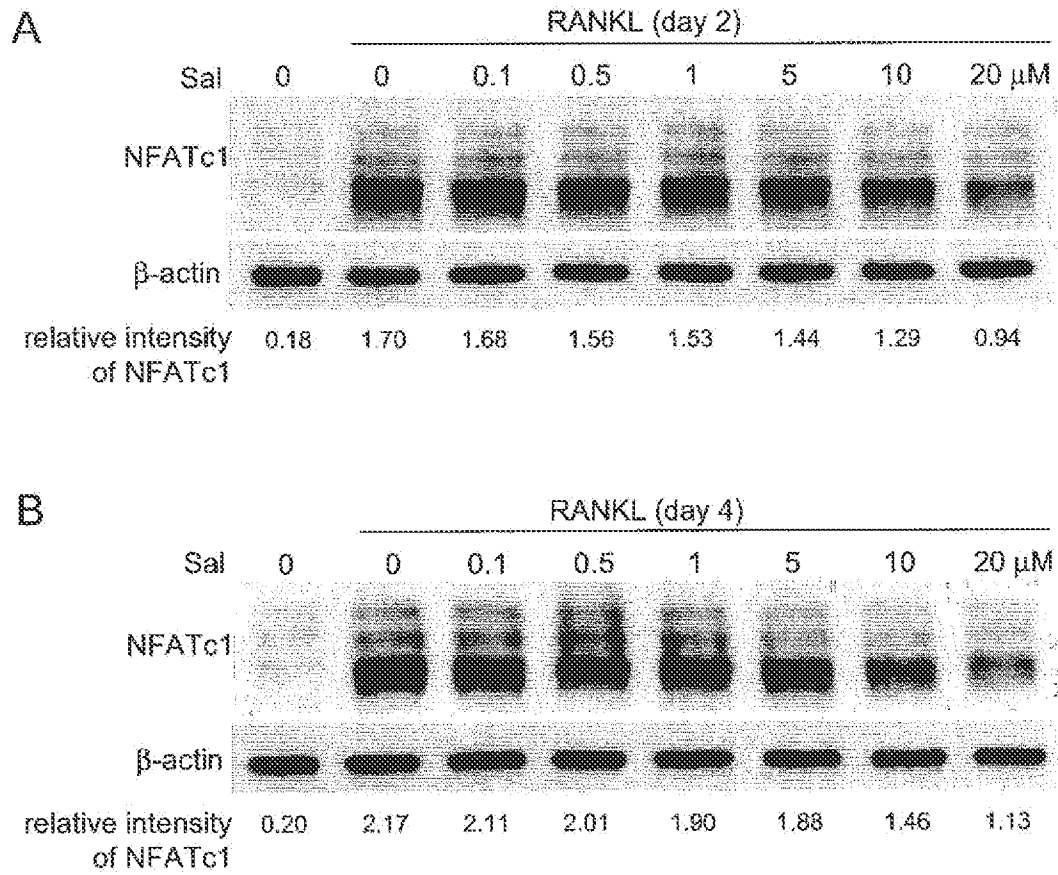


Figure 4

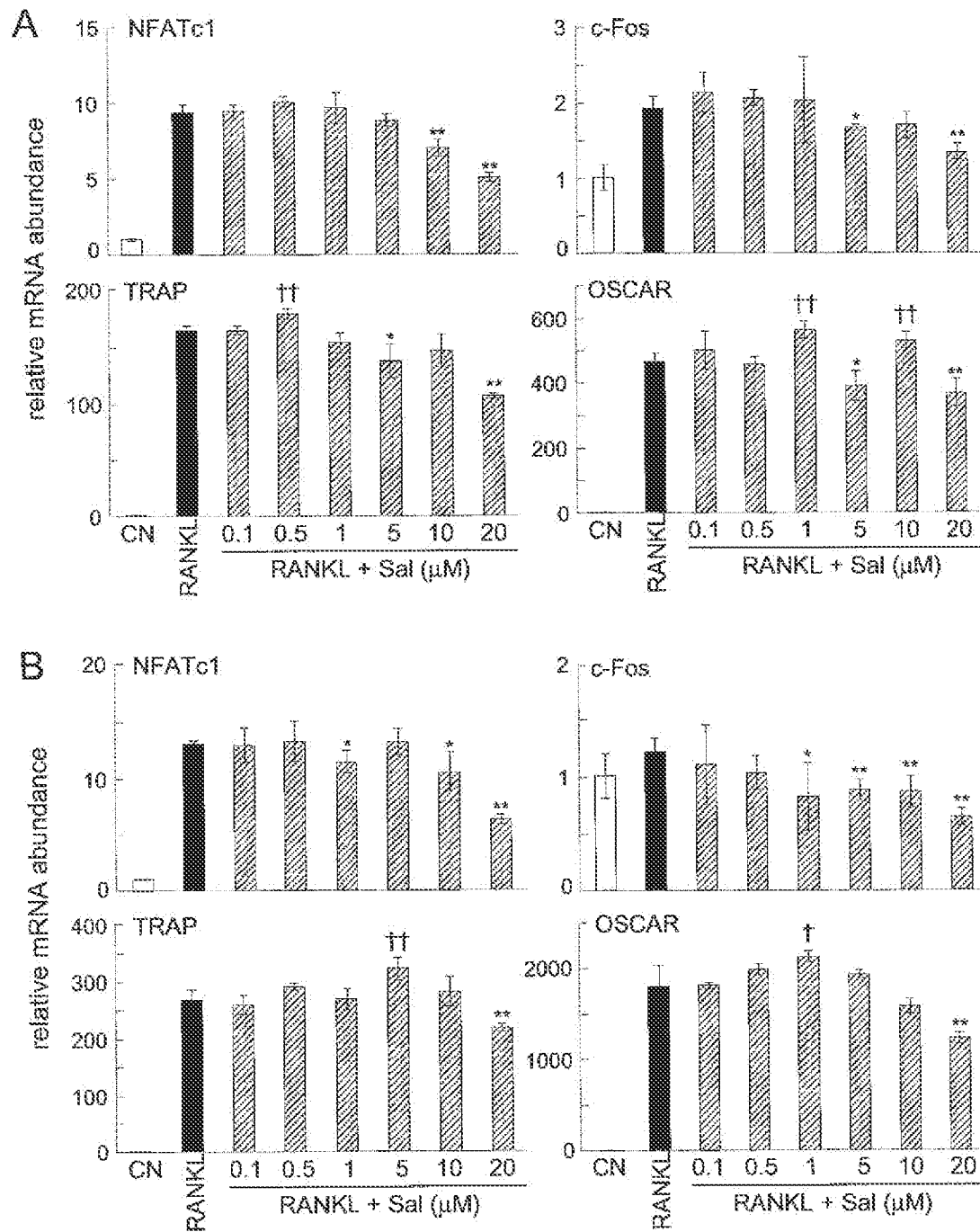


Figure 5

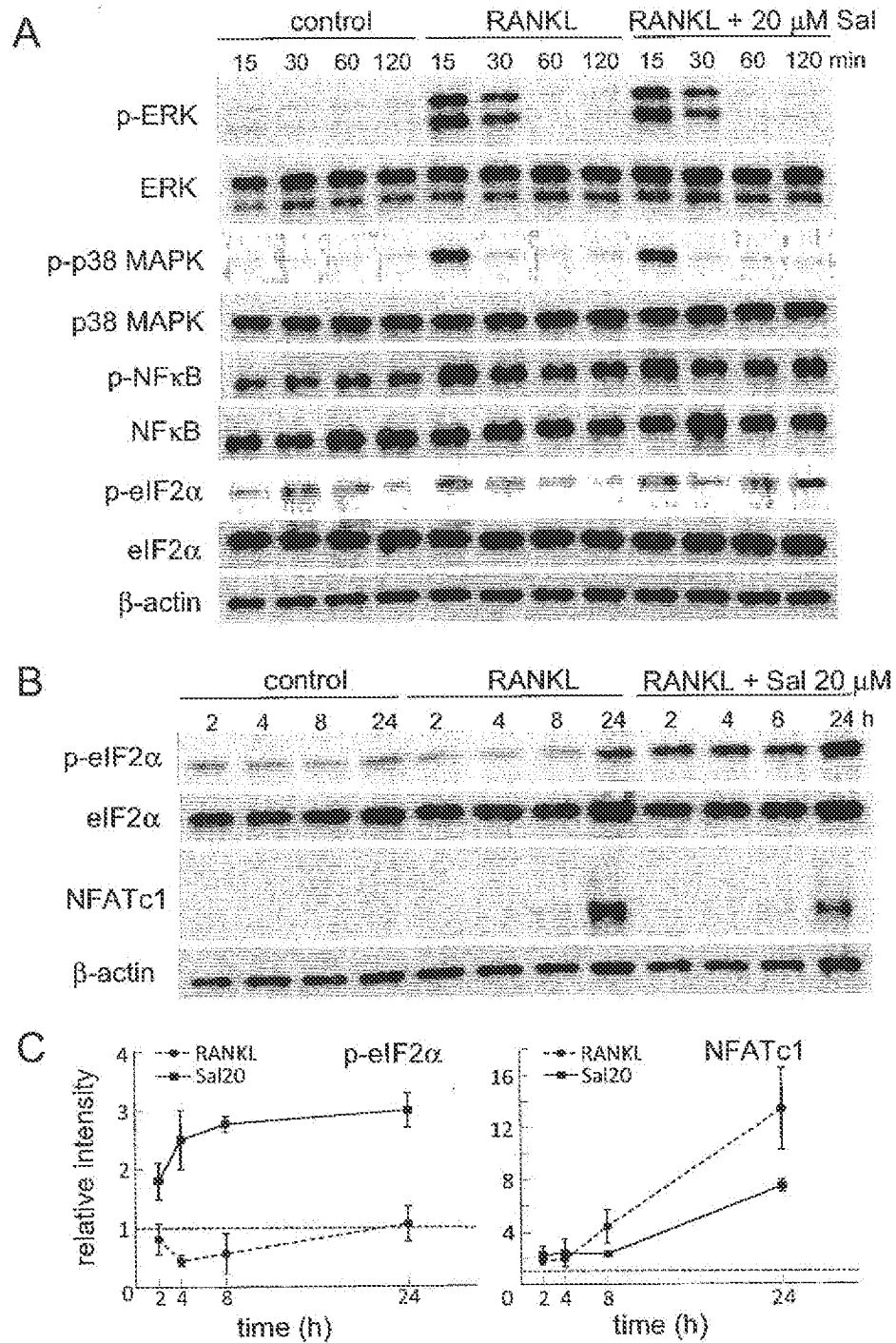


Figure 6

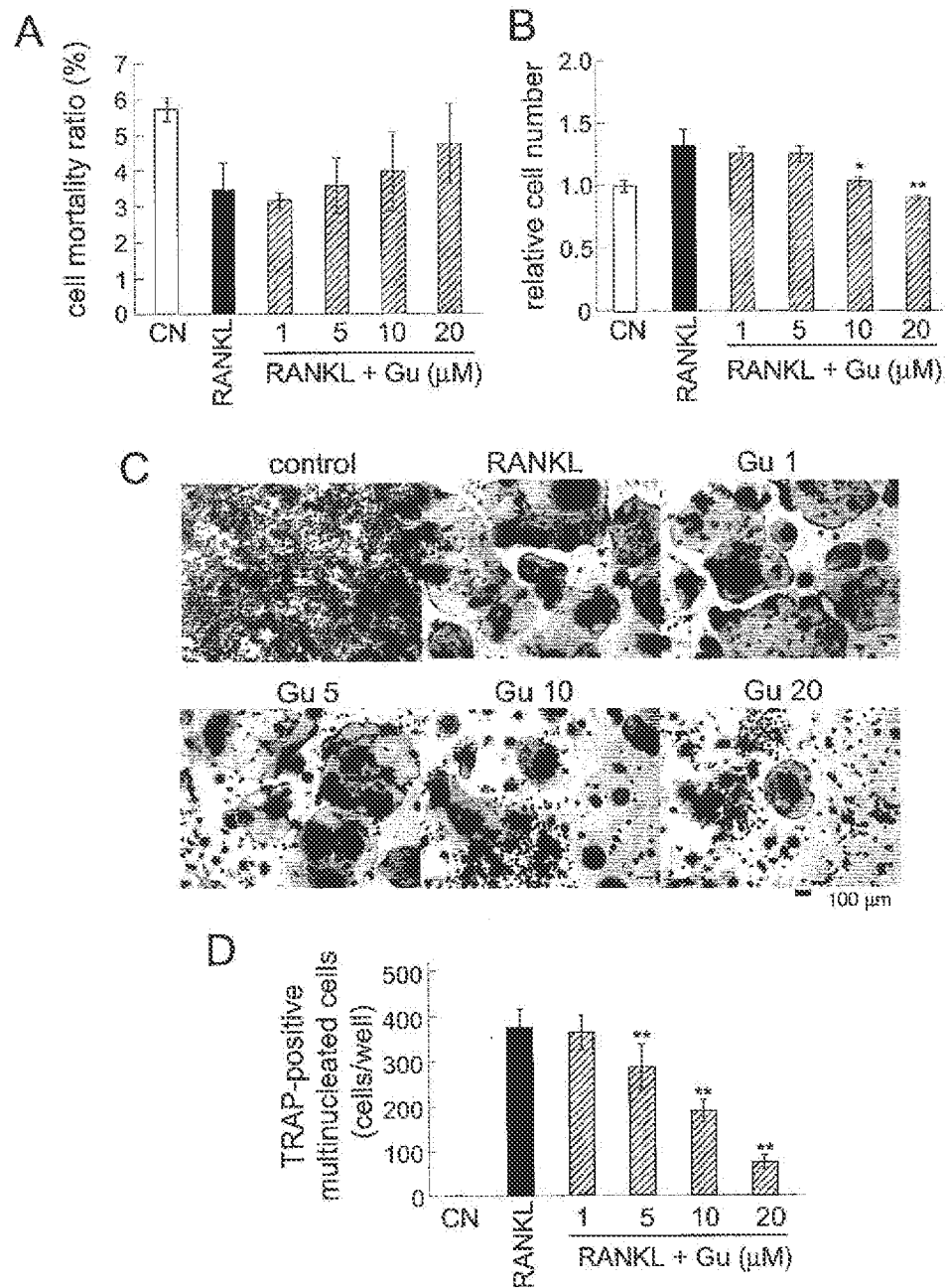


Figure 7

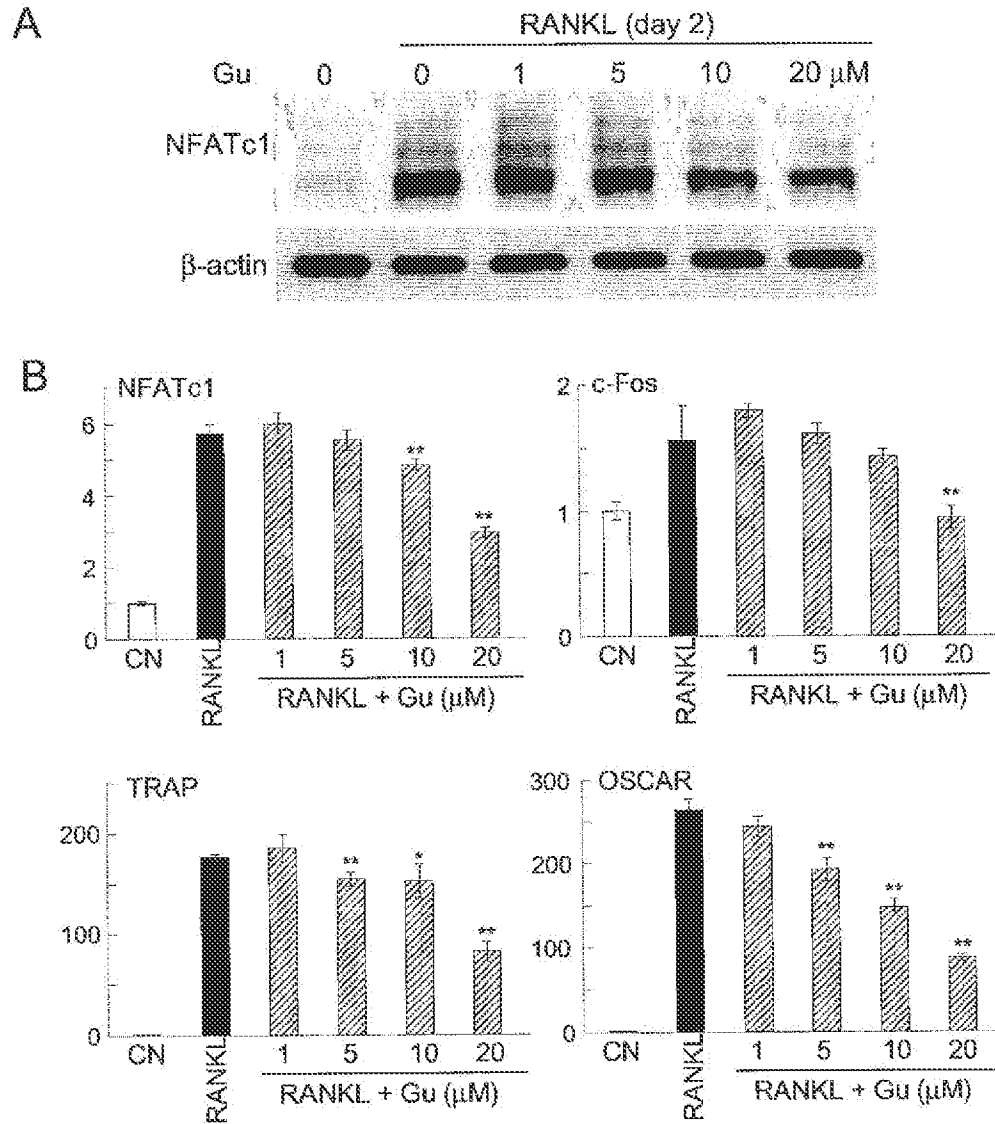


Figure 8

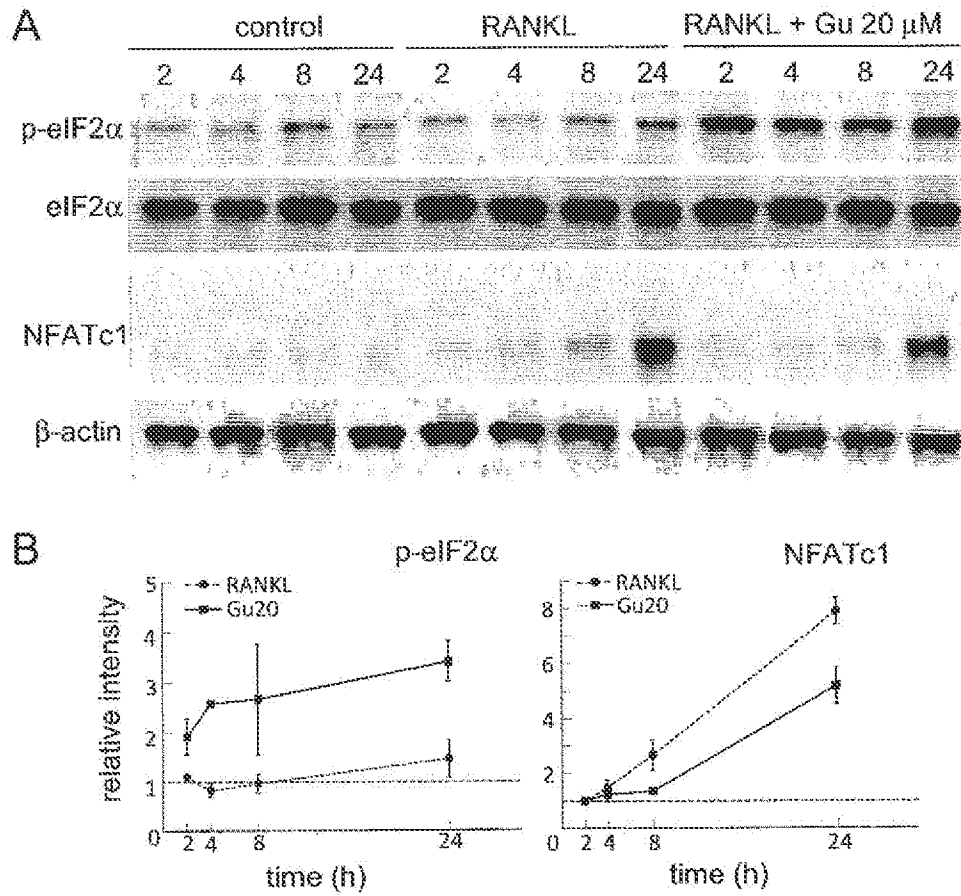


Figure 9

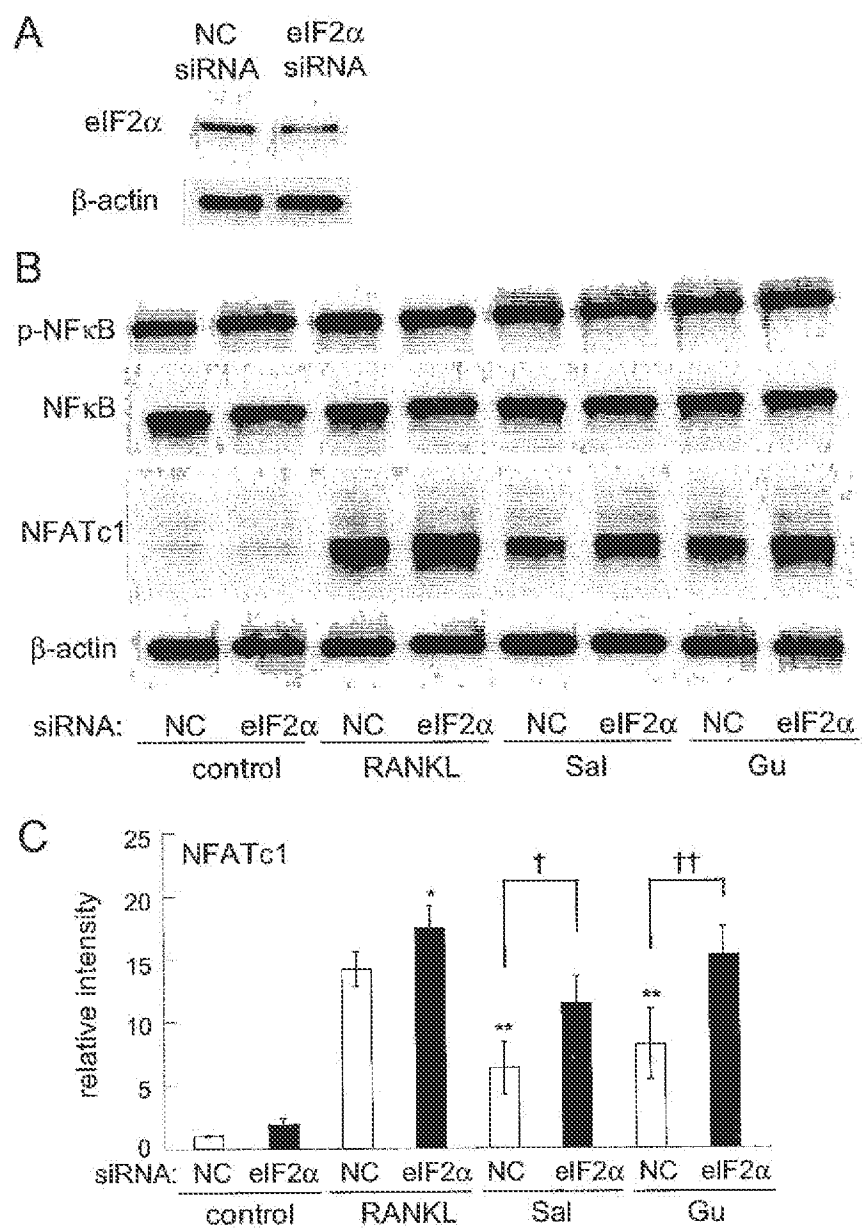


Figure 10

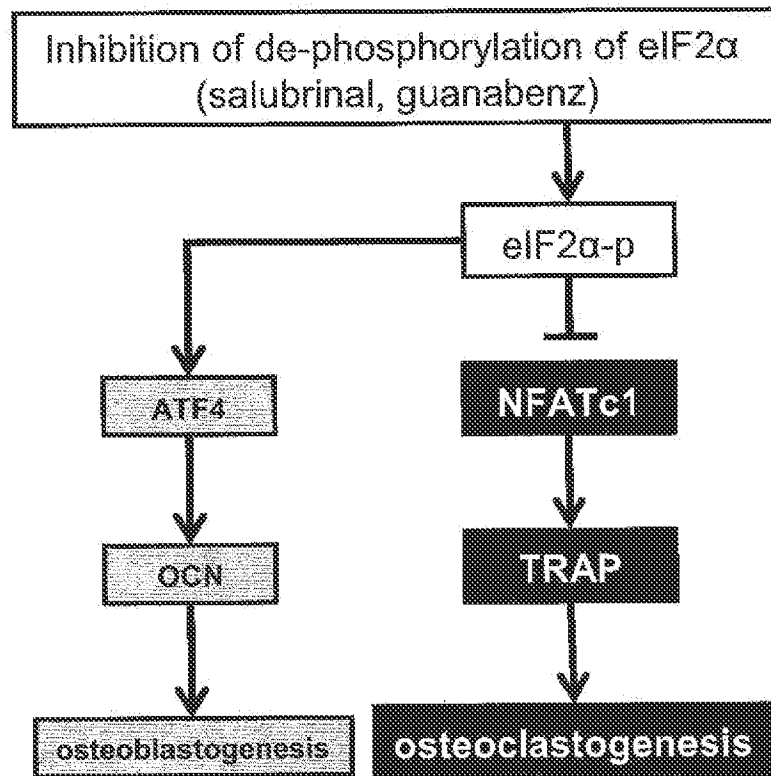


Figure 11

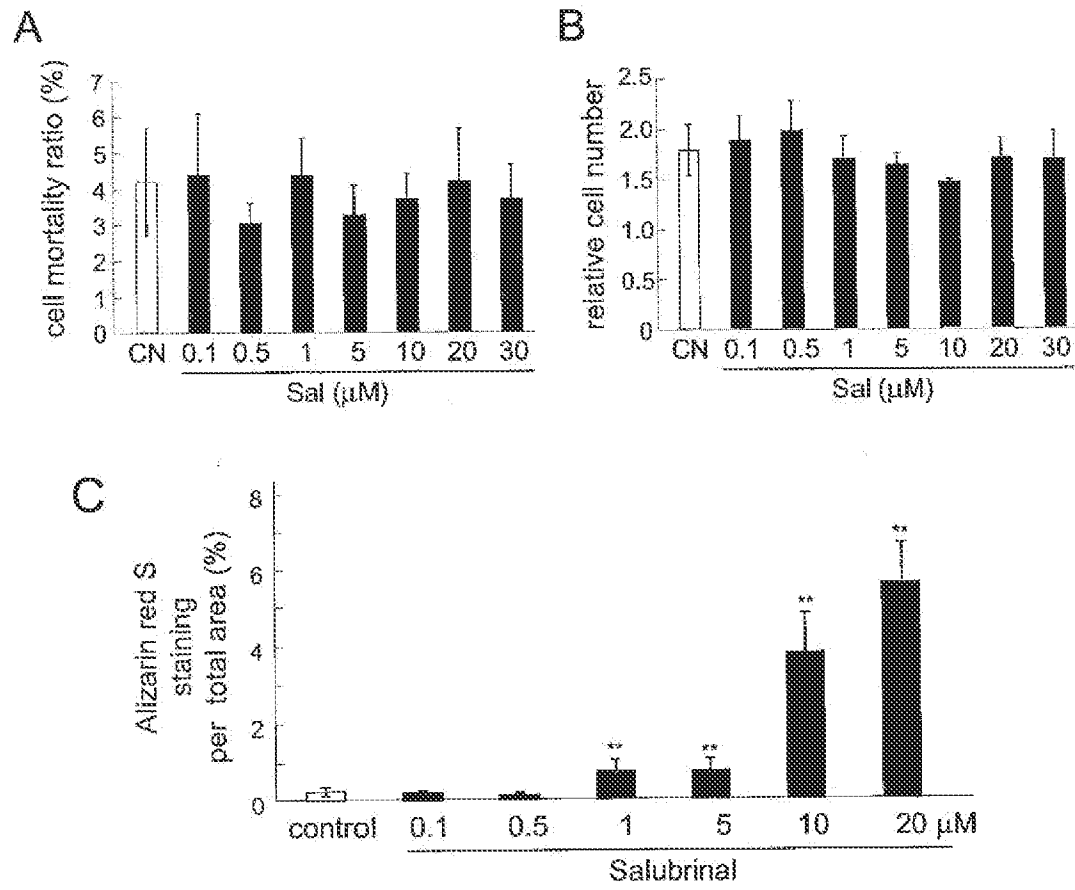


Fig. S1

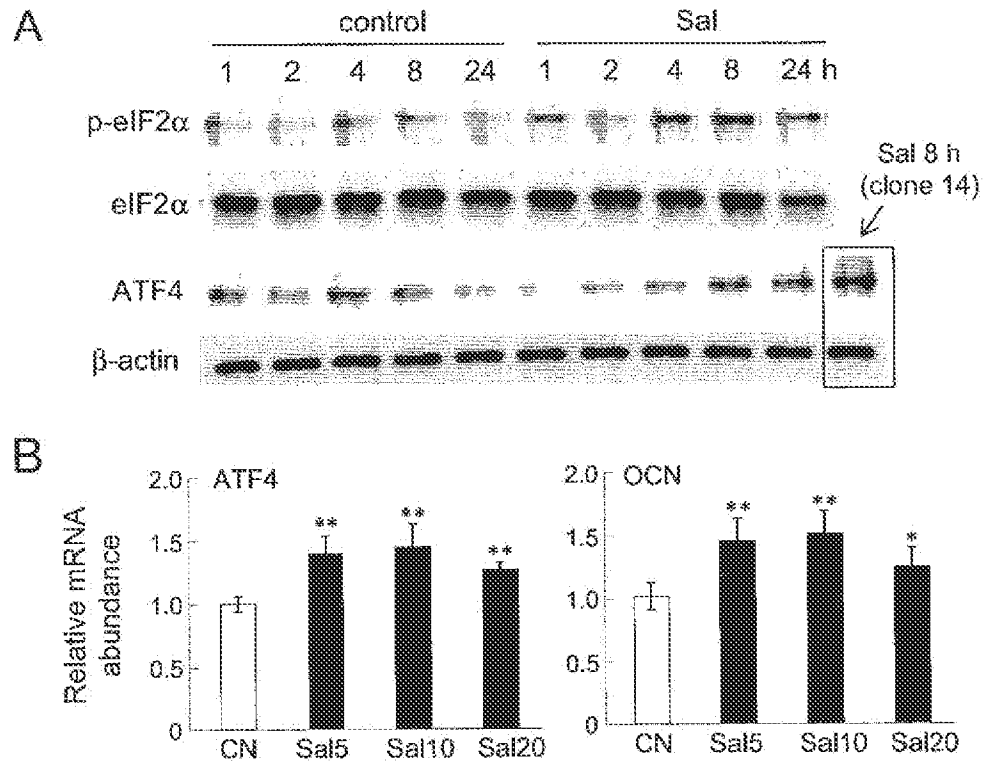


Fig. S2

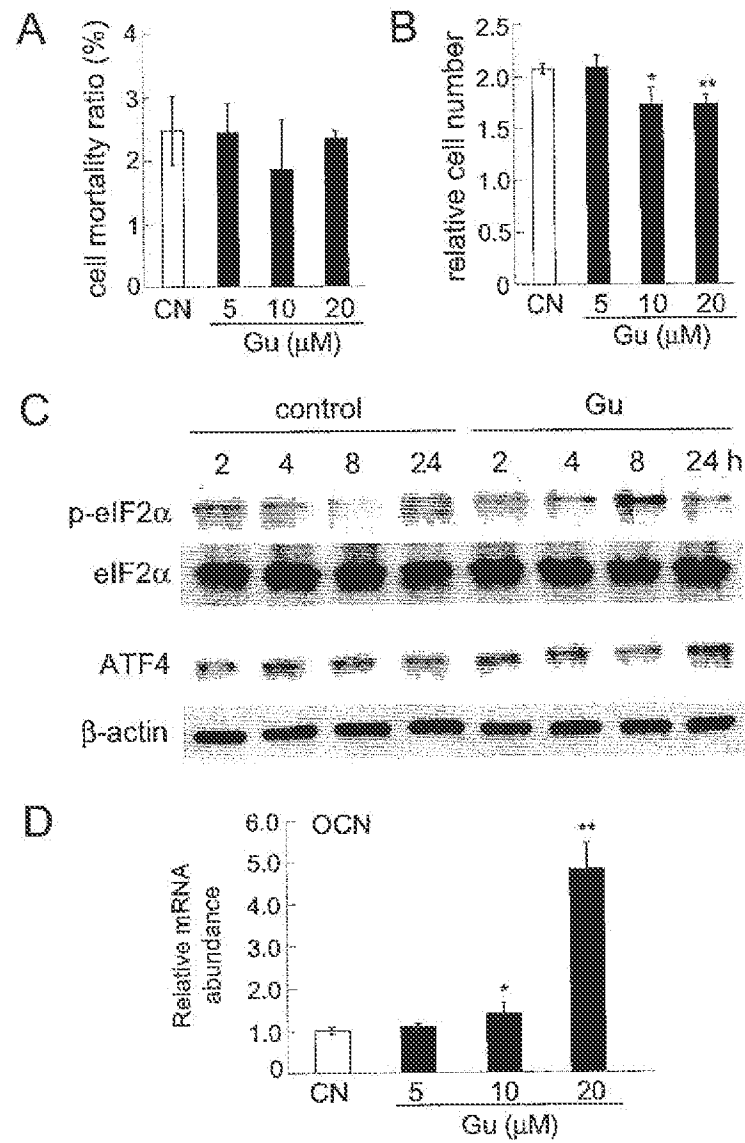


Fig. S3

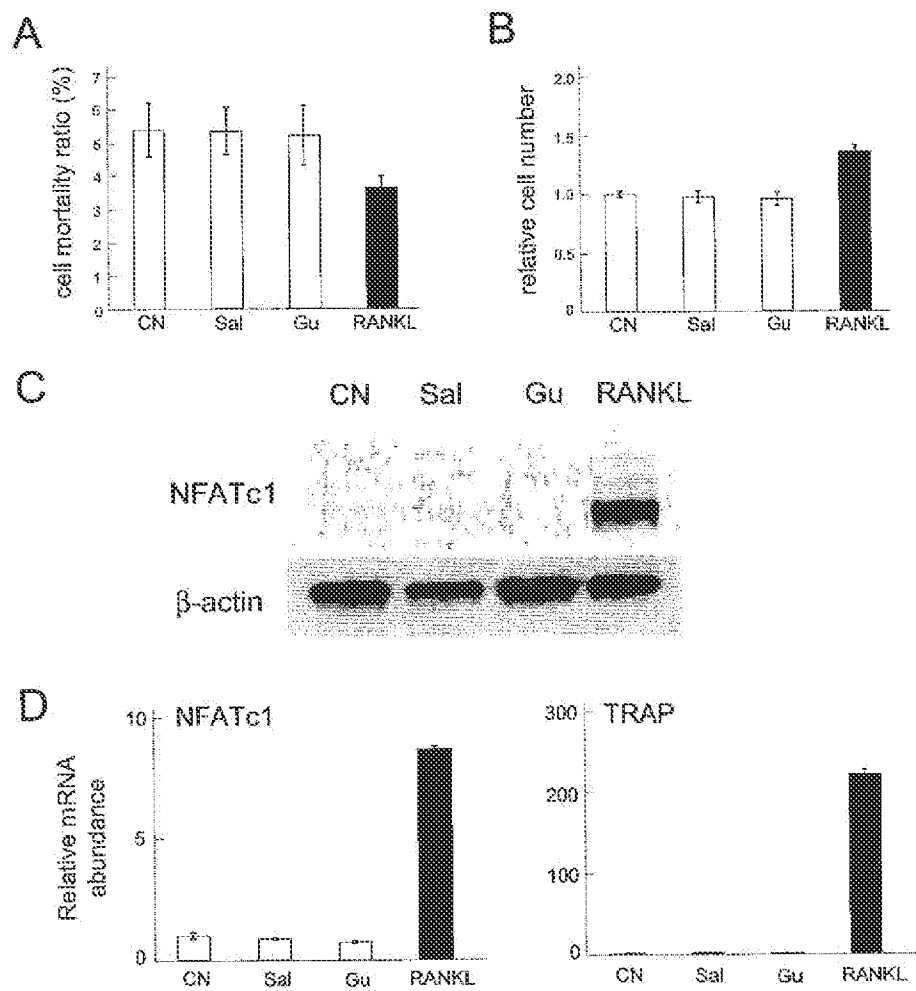


Fig. S4

ABSTRACT

Described herein are methods for treating bone diseases or defects. The methods include administering to a host animal therapeutically effective amounts of one or more compounds that are selective inhibitors of dephosphorylation of eIF2 α .

Appendix 2

Principal Component Analysis of the Regulation of Osteoclastogenesis by Salubrinal and Guanabenz

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Introduction: Osteoporosis is a common debilitating disease that causes bone loss. Salubrinal and guanabenz, new potential therapeutic agents for treating osteoporosis, have attracted attention since they not only activate bone-forming osteoblasts but also suppress bone-resorbing osteoclasts [1]. They are known to elevate ATF4, one of the known transcription factors for bone formation. However, the mechanism of their action on bone resorption is not fully understood. Focusing on regulation of NFATc1, a master transcription factor of osteoclast development, we analyzed the genome-wide expression data and predicted potential mediators of salubrinal and guanabenz on bone resorption.

Materials and Methods: Principal component analysis (PCA) was conducted [2] using two sets of genome-wide mRNA expression profiles with RAW264.7 mouse pre-osteoclast cells (Figure 1). In the first set (4 groups), cells were treated with placebo, RANKL (stimulator of osteoclastogenesis), salubrinal with RANKL, or guanabenz with RANKL. In the second set (4 groups), cells were treated with control siRNA or NFATc1 siRNA in the presence and absence of RANKL. First, we applied PCA and selected genes that were apparently not regulated by NFATc1, through a comparison between the control and NFATc1 siRNA samples in the presence of RANKL. The genes in the 10th percentile of NFATc1 siRNA-dependent expression, whose components in the first principal axis (PC1) were smaller than others, were chosen. For those genes, we applied PCA for identifying genes responsive to salubrinal and guanabenz. Responsiveness was evaluated based on the components in the first principal axis, which clustered the samples by the degree of osteoclastogenesis. In determining responsiveness, we considered statistical significance (root mean squared combined p-values) among the samples with and without salubrinal or guanabenz treatment.

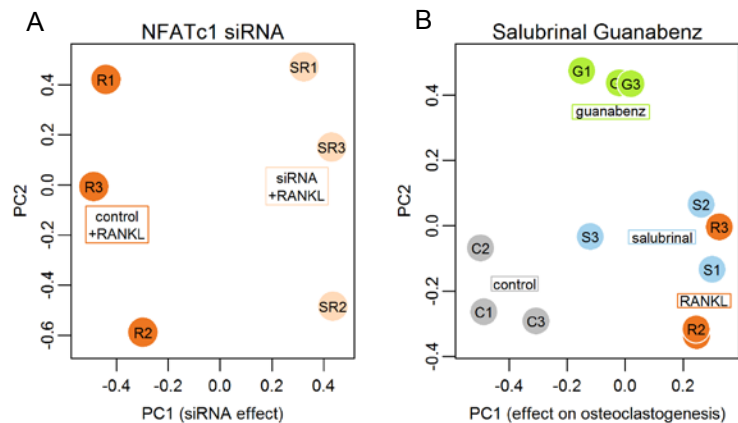


Figure 1. PCA on two data sets. (A) PC1 (first principal axis for siRNA) and PC2 (second principal axis) sort the samples based on the effects of NFATc1 siRNA. (B) PC1 for Sal/Gu clusters 4 sets of samples along the predicted axis of osteoclastogenesis.

Results and Discussion: PCA predicted that Serinc2, Rdx, and Antxr2 are potential activators of RANKL-mediated osteoclastogenesis, whereas Arl11, Zfyve21, and Pcdhb10 are potential inhibitors of RANKL-mediated osteoclastogenesis. These genes were identified to be regulated by salubrinal and guanabenz, but unaffected by the silencing of NFATc1.

Conclusions: In this study, we used PCA to predict potential regulators of salubrinal's and guanabenz's action on RANKL-mediated osteoclastogenesis. The predicted genes are upstream of NFATc1 or unrelated to NFATc1. Further validation of these candidates will be required in order to confirm their activity in regulating osteoclasts. The elucidation of these mechanisms will refine our understanding of osteoclastogenesis and may help direct future targets for drug treatment.

References:

- [1] Hamamura, K, et al. J Bone Miner Metab, 2013, vol. 31, pp. 618-628.
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Suppression of osteoclastogenesis through phosphorylation of eukaryotic translation initiation factor 2 alpha

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Abstract In response to various stresses including viral infection, nutrient deprivation, and stress to the endoplasmic reticulum, eukaryotic translation initiation factor 2 alpha (eIF2 α) is phosphorylated to cope with stress induced apoptosis. Although bone cells are sensitive to environmental stresses that alter the phosphorylation level of eIF2 α , little is known about the role of eIF2 α mediated signaling during the development of bone-resorbing osteoclasts. Using two chemical agents (salubrinal and guanabenz) that selectively inhibit de-phosphorylation of eIF2 α , we evaluated the effects of phosphorylation of eIF2 α on osteoclastogenesis of RAW264.7 pre-osteoclasts as well as development of MC3T3 E1 osteoblast-like cells. The result showed that salubrinal and guanabenz stimulated matrix deposition of osteoblasts through upregulation of activating transcription factor 4 (ATF4). The result also revealed that these agents reduced expression of the nuclear factor of activated T cells c1 (NFATc1) and inhibited differentiation of RAW264.7 cells to multi-nucleated osteoclasts. Partial silencing of eIF2 α with RNA interference reduced suppression of salubrinal/guanabenz-driven downregulation of NFATc1. Collectively, we demonstrated that the elevated phosphorylation level of

eIF2 α not only stimulates osteoblastogenesis but also inhibit osteoclastogenesis through regulation of ATF4 and NFATc1. The results suggest that eIF2 α -mediated signaling might provide a novel therapeutic target for preventing bone loss in osteoporosis.

Keywords Osteoclasts · Salubrinal · Guanabenz · eIF2 α · NFATc1

Introduction

Osteoblasts and osteoclasts are the two major types of bone cells in bone remodeling. Osteoblasts are bone-forming cells originated from mesenchymal stem cells, while osteoclasts are bone-resorbing cells derived from hematopoietic stem cells. These two types of cells orchestrate a complex remodeling process, in which mineralized bone matrix is degraded by osteoclasts and newly formed by osteoblasts [1, 2]. In order to maintain proper bone mass, exercise and calcium rich diets are recommended. However, a failure of the coordinated action such as in osteoporosis, which is a common form of bone loss prevailing among postmenopausal women, increases risk of bone fracture [3]. In order to develop therapeutic drugs for treatment of osteoporosis, an understanding of signaling pathways that govern osteoclastogenesis—development of pre-osteoclasts (monocyte/macrophage) to multi-nucleated osteoclasts—is required. In this paper, we examined a signaling pathway for osteoclastogenesis that is mediated by eukaryotic translation initiation factor 2 alpha (eIF2 α).

A protein complex, eIF2, is a heterotrimer essential for protein synthesis, and eIF2 α is one of its major components together with eIF2 β and eIF2 γ [4]. In response to various stresses such as oxidation, radiation, and stress to the

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endoplasmic reticulum that potentially lead to cellular apoptosis, a serine residue of eIF2 α is phosphorylated. This action would initiate a pro-survival program by lowering general translation efficiency except for a group of genes that includes activating transcription factor 4 (ATF4) [5]. The ATF4 is a transcription factor critical for osteoblastogenesis and bone formation [6]. In osteoblasts elevation of phosphorylated eIF2 α (p-eIF2 α) is reported to stimulate the expression of ATF4 [7, 8]. Little is known, however, about potential effects of p-eIF2 α on development of osteoclasts.

Herein we addressed a question: Does elevation of p-eIF2 α alter cellular fates of pre-osteoclasts? Osteoblasts and osteoclasts extensively interact through molecular pathways including RANK (receptor activator of nuclear factor kappa-B)/RANKL (RANK ligand)/OPG (osteoprotegerin) signaling [9, 10] and Wnt signaling [11]. Therefore, osteoclastogenesis is potentially regulated by signaling molecules that also affect osteoblastogenesis. Furthermore, osteoclastogenesis is influenced by various stresses such as estrogen deficiency and disuse or unloading [12]. Since elevation of p-eIF2 α can provide stress-relieving effects on osteoblasts, we hypothesized that elevation of p-eIF2 α suppresses differentiation of pre-osteoclasts to multi-nucleated osteoclasts.

In this study, we employed two chemical agents (salubrinal and guanabenz) and examined the effects of elevated p-eIF2 α on osteoclastogenesis. These two agents selectively inhibit de-phosphorylation of p-eIF2 α by interacting with protein phosphatase 1, PP1 [13, 14]. The signaling pathway, mediated by eIF2 α , is not directly linked to known agents for osteoclastogenesis such as calcium binding agents and RANKL. Currently, the most common medications, prescribed for preventing bone loss in patients with osteoporosis, are bisphosphonates. Bisphosphonates preferentially bind to calcium in bone and induce apoptosis of osteoclasts [15]. Other medications using neutralizing antibodies targeted to RANKL would block osteoclastogenesis by mimicking OPG's binding to RANKL [16]. The RANKL is a cytokine belonging to the tumor necrosis factor family, and is involved in T cell-dependent immune responses as well as differentiation and activation of osteoclasts [9, 10]. To our knowledge, no therapeutic agents for osteoporosis have been targeted to eIF2 α -mediated signaling.

We employed MC3T3 E1 osteoblast-like cells [17] and RAW264.7 cells [18] to evaluate osteoblastogenesis and osteoclastogenesis, respectively. In the presence and absence of salubrinal and guanabenz, MC3T3 E1 cells were cultured in an osteogenic medium for evaluation of matrix deposition, while RAW264.7 cells were cultured in an osteoclast differentiation medium for evaluation of multi-nucleation. Alizarin Red S staining was performed to

evaluate osteoblast mineralization for MC3T3 E1 cells, and TRAP staining was conducted to determine multi-nucleated osteoclasts proliferation for RAW264.7 cells. To analyze molecular signaling pathways, quantitative real-time PCR and Western blot analysis were conducted. The mRNA levels of ATF4, osteocalcin, c-Fos [19], tartrate-resistant acid phosphatase (TRAP) [20], and osteoclast-associated receptor (OSCAR) [21] were determined. The protein expression levels of eIF2 α , ATF4, and nuclear factor of activated T cells c1 (NFATc1) [22] were also determined. The NFATc1 is a transcription factor, which is critically important for development and activation of osteoclasts in response to RANKL. The RNA interference using siRNA specific to ATF4 and eIF2 α was conducted to evaluate the role of ATF4 in osteoblastogenesis and eIF2 α in osteoclastogenesis.

Materials and methods

Cell culture

The MC3T3 E1 mouse osteoblast-like cells (clone 14—MC3T3 E1-14; and no clonal cells in supplementary figures), and RAW264.7 mouse pre-osteoclast (monocyte/macrophage) cells were cultured in α MEM containing 10 % fetal bovine serum and antibiotics (50 U/ml penicillin, and 50 μ g/ml streptomycin; Life Technologies, Grand Island, NY, USA). Cells were maintained at 37 °C and 5 % CO₂ in a humidified incubator. Cell mortality and live cell numbers were determined 24 h after the treatment with 20 ng/ml RANKL (PeproTech, Rocky Hills, NC, USA) in response to 0.1–20 μ M salubrinal or 1–20 μ M guanabenz acetate (Tocris Bioscience, Ellisville, MO, USA). Cells were stained with trypan blue and the numbers of live and dead cells were counted using a hemacytometer.

Quantitative real-time PCR

Total RNA was extracted using an RNeasy Plus mini kit (Qiagen, Germantown, MD, USA). Reverse transcription was conducted with high capacity cDNA reverse transcription kits (Applied Biosystems, Carlsbad, CA, USA), and quantitative real-time PCR was performed using ABI 7500 with Power SYBR green PCR master mix kits (Applied Biosystems). We evaluated mRNA levels of ATF4, Osteocalcin (OCN), NFATc1, c-Fos, tartrate-resistant acid phosphatase (TRAP), and osteoclast-associated receptor (OSCAR) with the PCR primers listed in Table 1. The GAPDH was used for internal control. The relative mRNA abundance for the selected genes with respect to the level of GAPDH mRNA was expressed as a

Table 1 Real-time PCR primers used in this study

Target	Forward primer	Backward primer
ATF4	5'-TGGCGAGTGTAAAGGAGCTAGAAA-3'	5'-TCTTCCCCCTTGCCTTACG-3'
OCN	5'-CCGGGAGCAGTGTGAGCTTA-3'	5'-AGGCGGTCTTCAAGCCATACT-3'
NFATc1	5'-GGTGTGTCTGGCCATAACT-3'	5'-GCGGAAAGGTGGTATCTCAA-3'
c-Fos	5'-AGGCCAGTGGCTCAGAGA-3'	5'-CCAGTCTGCTGCATAGAAGGAA-3'
TRAP	5'-TCCTGGCTCAAAAAGCAGTT-3'	5'-ACATAGCCCACACCGTTCTC-3'
OSCAR	5'-ACACACACACCTGGCACCTA-3'	5'-GAGACCATCAAAGGCAGAGC-3'
GAPDH	5'-TGCACCACCAACTGCTTAG-3'	5'-GGATGCAGGGATGATGTTC-3'

ratio of $S_{\text{treated}}/S_{\text{control}}$, where S_{treated} is the mRNA level for the cells treated with chemical agents, and S_{control} is the mRNA level for control cells [23].

Western immunoblotting

Cells were lysed in a radioimmunoprecipitation assay (RIPA) buffer containing protease inhibitors (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phosphatase inhibitors (Calbiochem, Billerica, MA, USA). Isolated proteins were fractionated using 10–15 % SDS gels and electro-transferred to Immobilon-P membranes (Millipore, Billerica, MA, USA). The membrane was incubated for 1 h with primary antibodies followed by 45 min incubation with goat anti-rabbit or anti-mouse IgG conjugated with horseradish peroxidase (Cell Signaling, Danvers, MA, USA). We used antibodies against ATF4, NFATc1 (Santa Cruz), p-eIF2 α (Thermo Scientific, Waltham, MA, USA), eIF2 α , caspase 3, cleaved caspase 3, p38 and p-p38 mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and p-ERK, nuclear factor kappa B (NF κ B) p65 and p-NF κ B p65 (Cell Signaling), and β -actin (Sigma). Protein levels were assayed using a SuperSignal west femto maximum sensitivity substrate (Thermo Scientific), and signal intensities were quantified with a luminescent image analyzer (LAS-3000, Fuji Film, Tokyo, Japan).

Knockdown of ATF4 and eIF2 α by siRNA

Cells were treated with siRNA specific to ATF4 and eIF2 α (Life Technologies). Selected target sequences for knockdown of ATF4 and eIF2 α were: ATF4, 5'-GCU GCU UAC AUU ACU CUA A-3'; and eIF2 α , 5'-CGG UCA AAA UUC GAG CAG A-3'. As a nonspecific control, a negative siRNA (Silencer Select #1, Life Technologies) was used. Cells were transiently transfected with siRNA for ATF4, eIF2 α or control in Opti-MEM I medium with Lipofectamine RNAiMAX (Life Technologies). Six hours later, the medium was replaced by regular culture medium. The

efficiency of silencing was assessed with immunoblotting or quantitative PCR 48 h after transfection.

Mineralization assay

Mineralization of extracellular matrix was assayed by Alizarin Red S staining. The MC3T3-E1 cells were plated in 6-well plates. When cells were confluent, 50 μ g/ml of ascorbic acid (Wako Chemicals, Richmond, VA, USA) and 5 mM β -glycerophosphate (Sigma) were added. The medium was changed every other day, and staining was conducted after 3 weeks. Cells were washed with PBS twice and fixed with 60 % isopropanol for 1 min at room temperature, followed by rehydration with distilled water for 3 min at room temperature. They were stained with 1 % Alizarin red S (Sigma) for 3 min and washed with distilled water.

Osteoclastogenesis in vitro and TRAP (Tartrate-resistant acid phosphatase) staining

The RAW264.7 cells were plated at a density of 5×10^3 /cm² into a 12-well or a 60 mm dish, and cultured with 20 ng/ml RANKL in the presence and absence of salubrinal or guanabenz. The culture medium was replaced every 2 days. After 5 days of culture, the cells were stained for TRAP staining using an acid phosphatase leukocyte kit (Sigma). The number of TRAP-positive cells containing three or more nuclei was determined.

Statistical analysis

Three or four-independent experiments were conducted and data were expressed as mean \pm SD. For comparison among multiple samples, ANOVA followed by post hoc tests was conducted. Statistical significance was evaluated at $p < 0.05$. The single and double asterisks and daggers indicate $p < 0.05$ and $p < 0.01$. To determine intensities in immunoblotting and areas of Alizarin red S staining, images were scanned with Adobe Photoshop CS2 (Adobe Systems, San Jose, CA, USA) and quantified using Image J.

Results

Enhanced mineralization of MC3T3 E1-14 cells by salubrinal

Prior to examining the effects of salubrinal on osteoclastogenesis, we tested its effects on the development of osteoblasts focusing on cell viability, phosphorylation of eIF2 α (p-eIF2 α), expression of ATF4 and osteocalcin, and matrix mineralization. Administration of 5–20 μ M salubrinal to MC3T3 E1-14 cells did not increase cell mortality or inhibit cell proliferation (Fig. 1a). Unlike application of 10 nM thapsigargin, which is a stress inducer to the endoplasmic reticulum that elevates p-eIF2 α , incubation with 10 μ M salubrinal for 24 h did not elevate the expression level of cleaved caspase 3 (Fig. 1b). After 3-week incubation in an osteogenic medium, Alizarin red S staining area showed that salubrinal enhanced mineralization of MC3T3 E1-14 cells in a dose dependent manner (Fig. 1c). The enhanced mineralization was also observed in non-clonal MC3T3 E1 cells (Supplementary Fig. S1).

ATF4-mediated elevation of osteocalcin mRNA in MC3T3 E1-14 cells

Salubrinal is an inhibitor of de-phosphorylation of eIF2 α . Administration of 5 μ M salubrinal to MC3T3 E1-14 cells

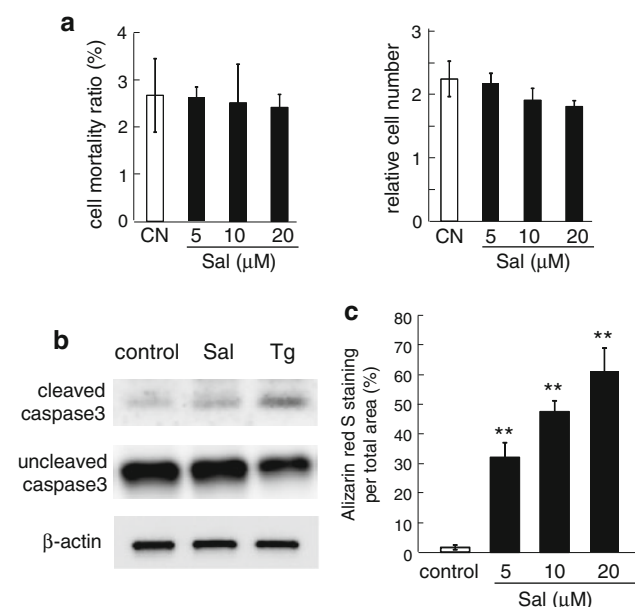


Fig. 1 Osteogenic effects of salubrinal on MC3T3 E1 (clone 14) osteoblast cells. *CN* control, *Sal* salubrinal, and *Tg* thapsigargin. The *double asterisk* indicates $p < 0.01$ in comparison to *CN*. **a** Cell mortality ratio and relative cell numbers. **b** No activation of cleaved caspase 3 by salubrinal. **c** Alizarin red S staining area in response to 5, 10, and 20 μ M salubrinal

elevated phosphorylation of eIF2 α , followed by an increase in ATF4 expression (Fig. 2a). Furthermore, the level of osteocalcin mRNA was increased 3.3 ± 0.5 fold (24 h) and 3.3 ± 0.3 fold (32 h) (Fig. 2b). When expression of ATF4 was significantly reduced by RNA interference (Fig. 2c, d), however, salubrinal-driven elevation of the osteocalcin mRNA level was suppressed (Fig. 2e). Non-clonal MC3T3 E1 cells also presented elevation of p-eIF2 α and ATF4, together with an increase in the mRNA levels of ATF4 and osteocalcin (Supplementary Fig. S2). In addition, administration of guanabenz to MC3T3 E1-14 elevated the mRNA level of osteocalcin in a dose dependent manner, consistent with an increase in p-eIF2 α and ATF4 (Supplementary Fig. S3).

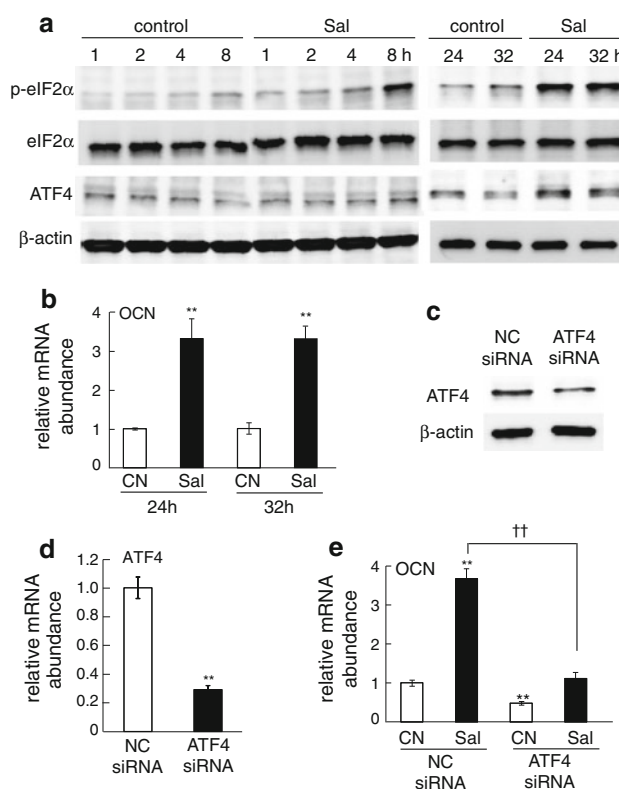


Fig. 2 Upregulation of p-eIF2 α , ATF4 and osteocalcin by salubrinal in MC3T3 E1 (clone 14) osteoblast cells in response to 5 μ M salubrinal. *CN* control, *Sal* salubrinal, and *NC* non-specific control siRNA. The *double asterisk* indicates $p < 0.01$ in comparison to *CN* or *NC*. The *double dagger* indicates with $p < 0.01$ in comparison to the salubrinal-treated *NC* siRNA cells. **a** Western blot analysis of p-eIF2 α and ATF4. **b** Salubrinal driven elevation of osteocalcin mRNA level. **c** ATF4 level after transfecting siRNA specific to ATF4. **d** Relative mRNA levels of ATF4 in response to RNA interference with ATF4 siRNA and non-specific control (*NC*) siRNA. **e** Relative mRNA levels of osteocalcin (*OCN*). The *asterisk* is for the comparison to the control with *NC* siRNA, and the *dagger* is the comparison between the samples transfected with ATF4 siRNA

Inhibition of osteoclastogenesis of RAW264.7 cells by salubrinal

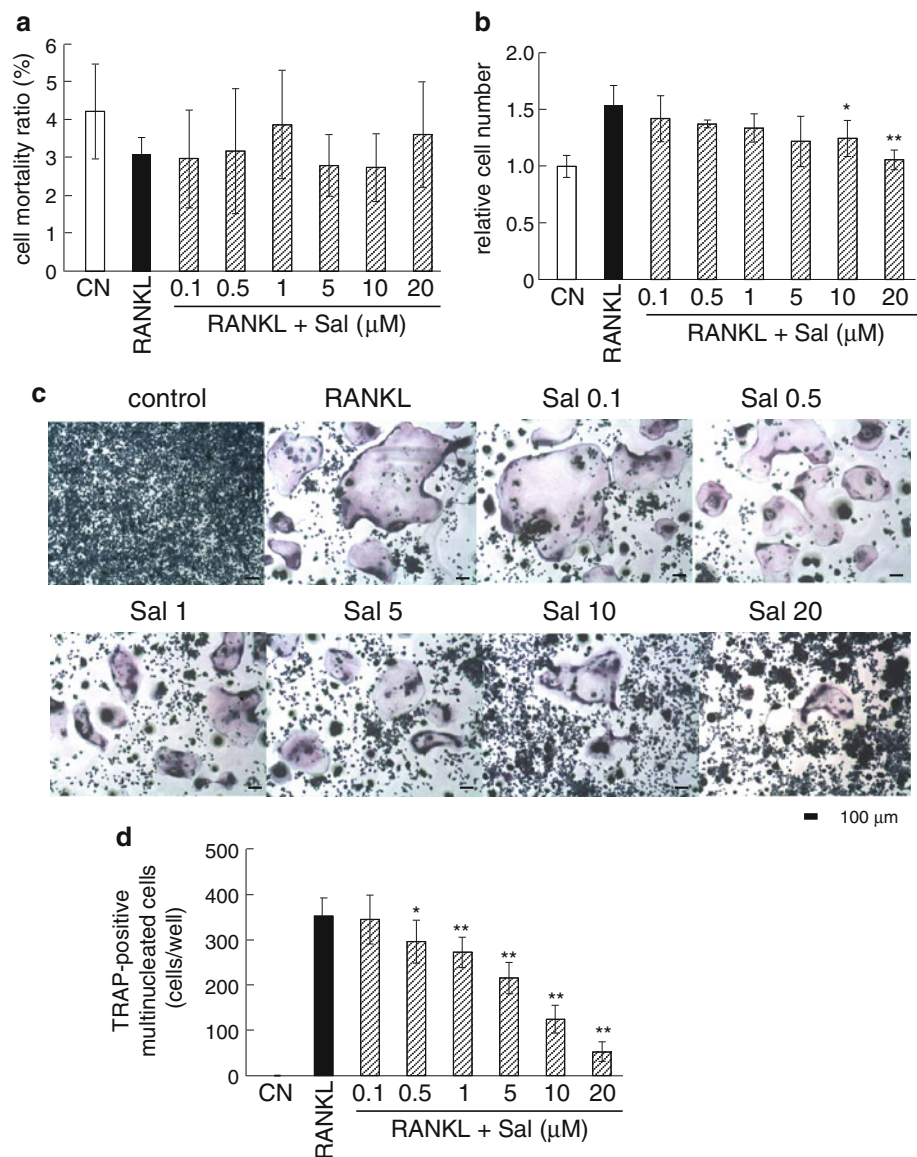
The primary aim of this study is to evaluate the effects of salubrinal on osteoclastogenesis. In response to 0.1–20 μM salubrinal for 24 h, we examined cell mortality and live cell numbers of RAW264.7 pre-osteoclasts. Cell mortality ratio did not present statistically significant differences in the presence and absence of RANKL (Fig. 3a). The number of live cells was increased by $\sim 50\%$ by incubation with RANKL, and administration of 10–20 μM salubrinal reduced the numbers approximately by 10% (Fig. 3b). Consistent with the stimulatory role of RANKL, the number of TRAP-positive multi-nucleated cells was substantially increased by the addition of RANKL. However,

administration of 0.5–20 μM salubrinal reduced the number of TRAP-positive cells in a dose dependent manner (Fig. 3c, d).

Downregulation of NFATc1 in RAW264.7 cells by salubrinal

The NFATc1 is a transcription factor critical for activating osteoclastogenesis. Addition of RANKL to the culture medium significantly induced NFATc1 expression at day 2 and maintained its elevated level on day 4 (Fig. 4). The RANKL-induced expression of NFATc1 was reduced by administration of 5–20 μM salubrinal on both days, and the effect of salubrinal was dose dependent (Fig. 4).

Fig. 3 Inhibitory effects of salubrinal on RAW264.7 pre-osteoclasts. *CN* control, and *Sal* salubrinal. The single and double asterisks indicate $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. **a** Cell mortality ratio. **b** Relative cell numbers. **c** Dose-dependent suppression of RANKL driven activation of osteoclasts by salubrinal. **d** Dose-dependent suppression of TRAP-positive multi-nucleated cells by salubrinal



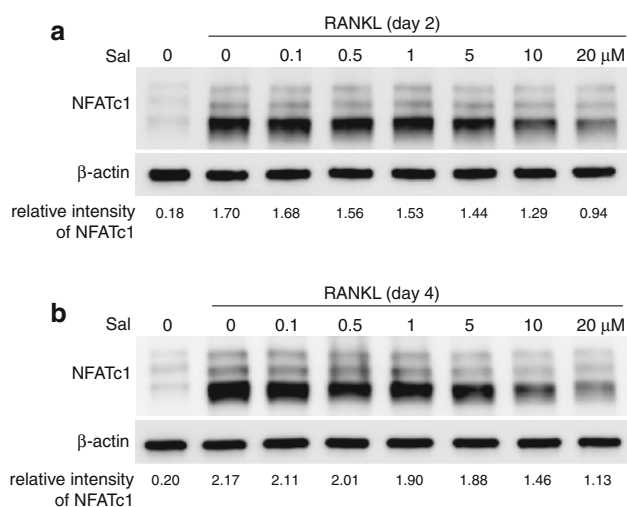


Fig. 4 Reduction of RANKL-induced NFATc1 expression by salubrinal. *Sal* salubrinal. The relative intensity of NFATc1 to β-actin is shown. **a** Expression of NFATc1 (2 days after RANKL administration). **b** Expression of NFATc1 (4 days after RANKL administration)

Partial suppression of mRNA levels of NFATc1, c-Fos, TRAP, and OSCAR by salubrinal

Addition of RANKL increased the mRNA levels of NFATc1, c-Fos, TRAP, and OSCAR, and administration of 20 μM salubrinal significantly reduced their mRNA levels. On day 2, for instance, the RANKL-driven increase was 9.4 ± 0.5 fold (NFATc1), 1.9 ± 0.1 fold (c-fos), 165 ± 4.2 fold (TRAP), and 467 ± 22 fold (OSCAR). The reduction by 20 μM salubrinal was 46 % (NFATc1), 32 % (c-fos), 35 % (TRAP), and 21 % (OSCAR) (Fig. 5a). Consistent with the observed dose response, administration of salubrinal at 0.1–1 μM did not contribute to significant reduction in these mRNA levels except for NFATc1 and c-fos on day 4 (Fig. 5b).

Temporal profile of p-eIF2α and NFATc1

The temporal expression profile revealed that addition of RANKL transiently reduced the phosphorylation level of eIF2α (2–8 h) and elevated NFATc1 by 13.4 ± 3.2 fold (24 h) (Fig. 6). This induction of NFATc1 was partially suppressed by salubrinal with an increase in the level of p-eIF2α. In the early period (2–4 h), administration of 20 μM salubrinal increased the level of p-eIF2α but did not alter the level of NFATc1. In the later period (8–24 h), however, the level of NFATc1 was significantly reduced by 48 % (8 h) and 44 % (24 h). Administration of 20 μM salubrinal did not significantly alter the phosphorylation level of ERK, p38 MAPK, and NFκB (Fig. 6). Note that the normalized level of “1” in Fig. 6c was defined as the

level for the cells that were not treated with RANKL without administration of guanabenz.

Inhibitory effects of guanabenz on osteoclastogenesis of RAW264.7 cells

To further examine a potential involvement of p-eIF2α in regulation of osteoclastogenesis, we employed guanabenz that also acts as an inhibitor of de-phosphorylation of eIF2α. Administration of 1 and 5 μM guanabenz did not alter cell mortality and the number of live cells, although its administration at 10 and 20 μM reduced the number of live cells in 24 h (Fig. 7a, b). Consistent with salubrinal’s inhibitory action, guanabenz also attenuated osteoclastogenesis of RAW264.7 cells in a dose dependent manner (Fig. 7c, d). Compared to the number of TRAP-positive multi-nucleated cells of 377 ± 39 (RANKL only), guanabenz reduced the number of differentiated osteoclasts to 364 ± 38 (1 μM), 288 ± 51 (5 μM), 189 ± 25 (10 μM), and 73 ± 16 (20 μM).

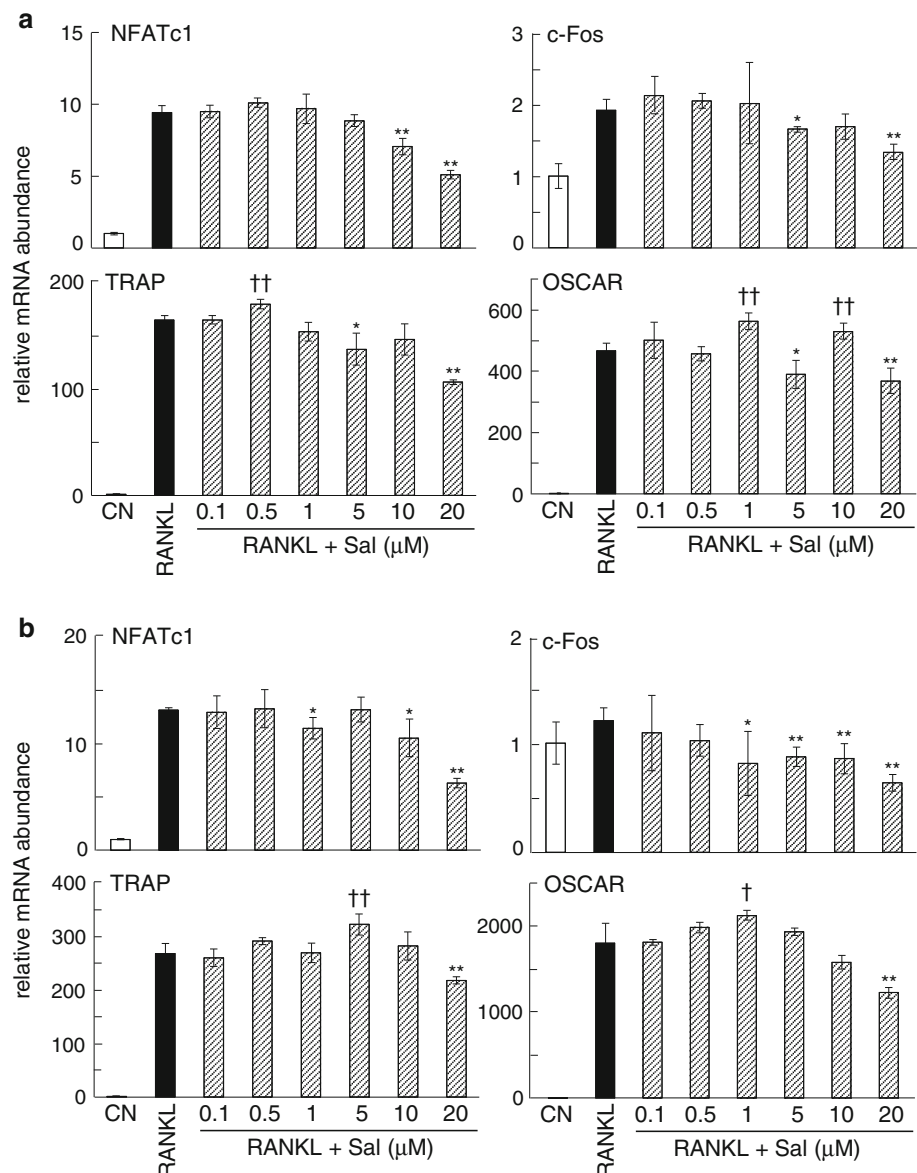
Reduction of RANKL-induced NFATc1, c-Fos, TRAP, and OSCAR by guanabenz

The induction of NFATc1 by RANKL was suppressed by guanabenz in a dose dependent manner (Fig. 8a). The mRNA levels of NFATc1, c-Fos, TRAP, and OSCAR were also reduced by administration of 20 μM guanabenz. Lower concentrations of guanabenz, 5 and 10 μM, were effective in reducing the levels of TRAP and OSCAR mRNA (Fig. 8b). The temporal expression profile of p-eIF2α and NFATc1 in response to 20 μM guanabenz revealed that p-eIF2α was upregulated in 2 h and NFATc1 was partially suppressed in 8 h (Fig. 9). The normalized level of “1” was defined as the level for the cells that were not treated with RANKL without administration of guanabenz. In the absence of RANKL administration, however, either salubrinal or guanabenz did not significantly alter cell mortality and expression of NFATc1 and TRAP (Supplementary Fig. S4).

Reduction in salubrinal/guanabenz-driven suppression of NFATc1 expression by RNA interference for eIF2α

To evaluate the effects of eIF2α on the expression level of NFATc1, we employed RNA interference specific for eIF2α together with a non-specific control (NC) (Fig. 10). In response to 20 μM salubrinal, RAW264.7 cells transfected with the control siRNA demonstrated a reduction of NFATc1 by 56 %. However, the expression of NFATc1 was reduced only by 20 % in the cells transfected with eIF2α siRNA. Furthermore, 20 μM guanabenz decreased the level of NFATc1 by 43 % in the cells transfected with

Fig. 5 Effects of salubrinal on mRNA expression levels of NFATc1, c-Fos, TRAP, and OSCAR. *CN* control. The *single and double asterisks* indicate significant decreases with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. The *single and double daggers* indicate significant increases with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. **a** Messenger RNA levels (2 days after RANKL administration). **b** Messenger RNA levels (4 days after RANKL administration)



the control siRNA but the transfection of eIF2 α siRNA abolished the suppressive effect of guanabenz. The phosphorylation level of NF κ B was not significantly altered by transfection with eIF2 α siRNA.

Discussion

In this study we demonstrate that differentiation of RAW264.7 pre-osteoclasts to multi-nucleated osteoclasts is inhibited by administration of salubrinal and guanabenz, which block de-phosphorylation of eIF2 α and elevate the level of p-eIF2 α . The growth area covered by multi-nucleated cells is significantly reduced by salubrinal and

guanabenz in a dose dependent manner. Partially silencing eIF2 α using RNA interference significantly suppressed salubrinal/guanabenz-driven reduction of NFATc1 expression. Together with the stimulated development of MC3T3 E1 osteoblasts by an increase in ATF4 expression, the results herein suggest that eIF2 α mediated signaling may play a physiological role in osteoclastogenesis and osteoblastogenesis.

Both salubrinal and guanabenz interact with PP1 and inhibit its activity of de-phosphorylating p-eIF2 α . Guanabenz is reported to bind to PP1R15A subunit [14], while the exact binding site of salubrinal is not known. Guanabenz is also known as an α_2 -adrenergic receptor agonist and used to treat hypertension [24]. The observed

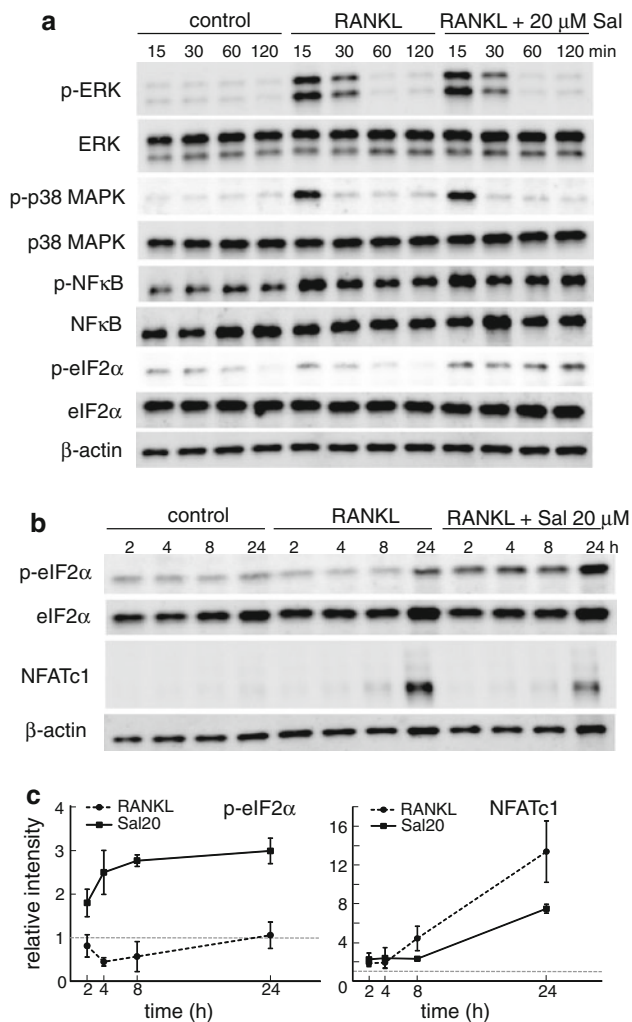


Fig. 6 Temporal expression profile of p-ERK, p-p38 MAPK, p-NFκB, p-eIF2α and NFATc1 in the presence and absence of 20 μM salubrinal. **a** Western blot analysis of p-ERK, p-p38 MAPK, p-NFκB, and p-eIF2α at 15, 30, 60 and 120 min. **b** Western blot analysis of eIF2α-p and NFATc1. **c** Comparison of the expression level of eIF2α-p and NFATc1 with and without 20 μM salubrinal. The normalized level of "1" was defined as the level for the cells that were not treated with RANKL without administration of salubrinal

stimulation of osteoblastogenesis as well as attenuation of osteoclastogenesis by both agents strongly indicates that eIF2α-mediated signaling is central to regulation of ATF4 and NFATc1. This result is also supported by the salubrinal-driven alterations in the mRNA levels of osteocalcin and TRAP, which are representative in development of osteoblasts and osteoclasts, respectively. Osteocalcin is synthesized solely by osteoblasts for matrix mineralization and calcium homeostasis [25], while TRAP is highly expressed in osteoclasts and its overexpression has been observed to cause bone loss in transgenic mice [26].

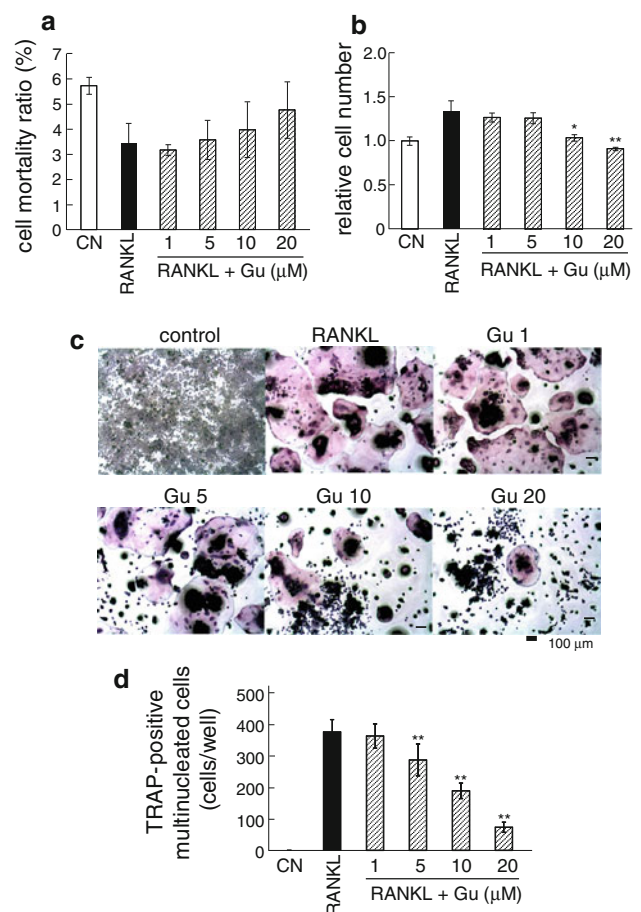


Fig. 7 Inhibitory effects of guanabenz on development of RAW264.7 pre-osteoclasts. CN control, Gu guanabenz. The single and double asterisks indicate significant decreases with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. **a** Cell mortality ratio. **b** Relative cell numbers. **c** Dose-dependent suppression of RANKL driven activation of osteoclasts by guanabenz. **d** Dose-dependent suppression of TRAP-positive multi-nucleated cells by guanabenz

The elevation of p-eIF2α is reported to enhance the development of osteoblasts and mineralization of extracellular matrix. In response to various stresses such as oxidation, radiation, and stress to the endoplasmic reticulum, cells undergo either survival or an apoptotic pathway [27]. As part of a pro-survival program, the level of p-eIF2α is raised followed by diminished translational efficiency to all proteins except for a limited group including ATF4 [5]. Salubrinal's action mimics the induction of a pro-survival program without imposing damaging stresses, which result in the upregulation of ATF4 without inducing apoptosis. Since ATF4 is a transcription factor critical for osteoblastogenesis and bone formation, we examined the effects of the administration of

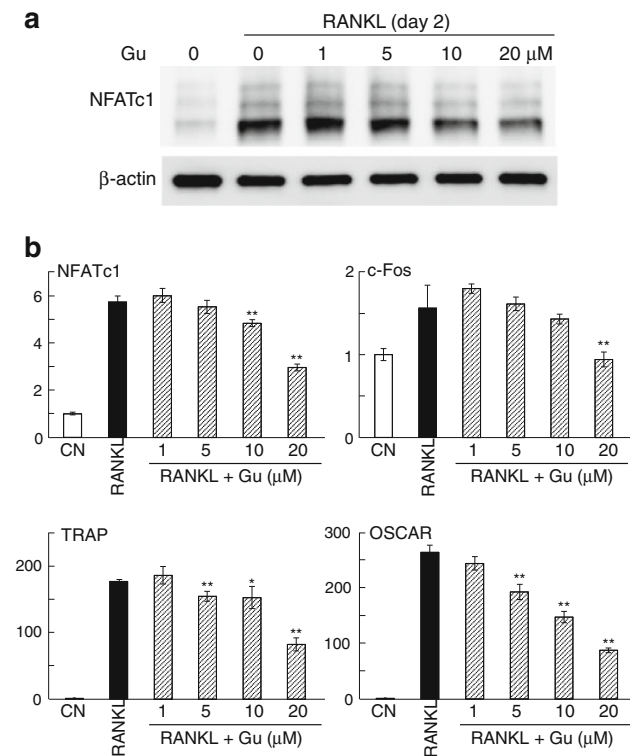


Fig. 8 Reduction of RANKL-induced NFATc1 expression by guanabenz. CN control, Gu guanabenz. The single and double asterisks indicate significant decreases with $p < 0.05$ and $p < 0.01$ in comparison to the RANKL-treated cells, respectively. **a** Expression of NFATc1 (2 days after RANKL administration). **b** Messenger RNA levels of NFATc1, c-Fos, TRAP, and OSCAR (2 days after RANKL administration)

salubrinal and guanabenz on the mRNA level of osteocalcin as well as the mineralization of the extracellular matrix. Silencing ATF4 using RNA interference significantly suppressed salubrinal-driven upregulation of osteocalcin expression. Thus, the result here is consistent with the previously reported role of salubrinal that stimulates new bone formation in the healing of bone wound [8].

A schematic diagram illustrating the proposed pathway of eIF2 α -mediated signaling in osteoblastogenesis and osteoclastogenesis is presented (Fig. 11). Through inhibition of de-phosphorylation of eIF2 α , salubrinal and guanabenz are capable of enhancing bone formation by activating ATF4, as well as reducing bone resorption by down-regulating NFATc1. Osteoclastogenesis is a complex developmental process, in which active interactions take place between osteoblasts and osteoclasts. In the RANK/RANKL/OPG signaling pathway, for instance, osteoblasts provide RANKL that stimulates osteoclastogenesis. Since binding of RANKL to RANK is known to activate MAPKs and NF κ B [28, 29], we evaluated a potential role of ERK, p38, and NF κ B in the eIF2 α -mediated signaling. In

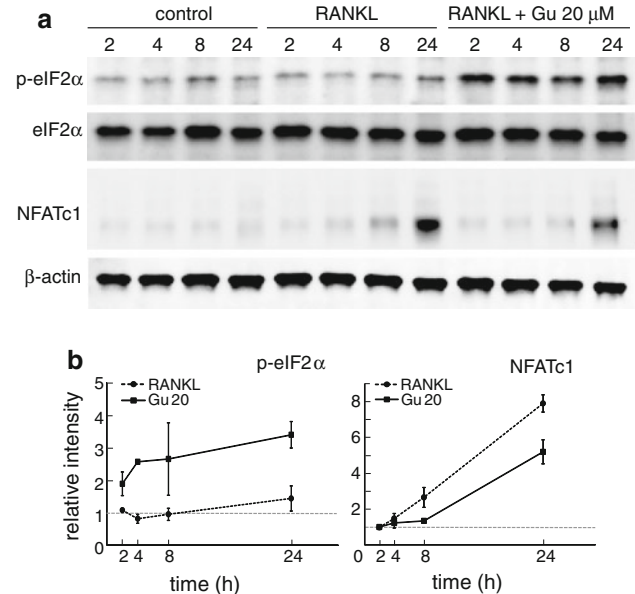


Fig. 9 Temporal expression profile of p-eIF2 α and NFATc1 in the presence and absence of 20 μ M guanabenz. Gu guanabenz. **a** Western blot analysis of eIF2 α -p and NFATc1. **b** Comparison of the expression level of eIF2 α -p and NFATc1 with and without 20 μ M guanabenz. The normalized level of “1” was defined as the level for the cells that were not treated with RANKL without administration of guanabenz

response to administration of 20 μ M salubrinal, we examined the levels of p-ERK, p-p38 MAPK, and p-NF κ B together with p-eIF2 α . However, no detectable changes in the levels of their phosphorylated form were observed. It is possible that salubrinal may activate transcription factors such as MafB (V-maf musculoaponeurotic fibrosarcoma oncogene homolog B), IRF8 (interferon regulatory factor 8), and Bcl6 (B cell lymphoma 6), which are known to be inhibitors of NFATc1 [30–32]. Alternatively, microRNA and epigenetic changes such as histone modification regulate expression of NFATc1 might be involved [33, 34]. For instance, H3K27 demethylase is reported to demethylate the site of H3K27me3 of NFATc1 and stimulate RANKL-induced osteoclastogenesis [34]. The results herein require further analysis on a regulatory mechanism that links elevation of p-eIF2 α to the suppression of NFATc1.

A recent study independently reported that salubrinal alters the fate of osteoclasts and bone resorption through eIF2 α -mediated translational regulation [35]. Herein, we further examined the regulatory mechanism using not only salubrinal but also guanabenz, which are the inhibitors of PP1. The results revealed that these agents can also regulate expression of NFATc1 at a transcriptional level. A separate in vivo study as well as in vitro studies using

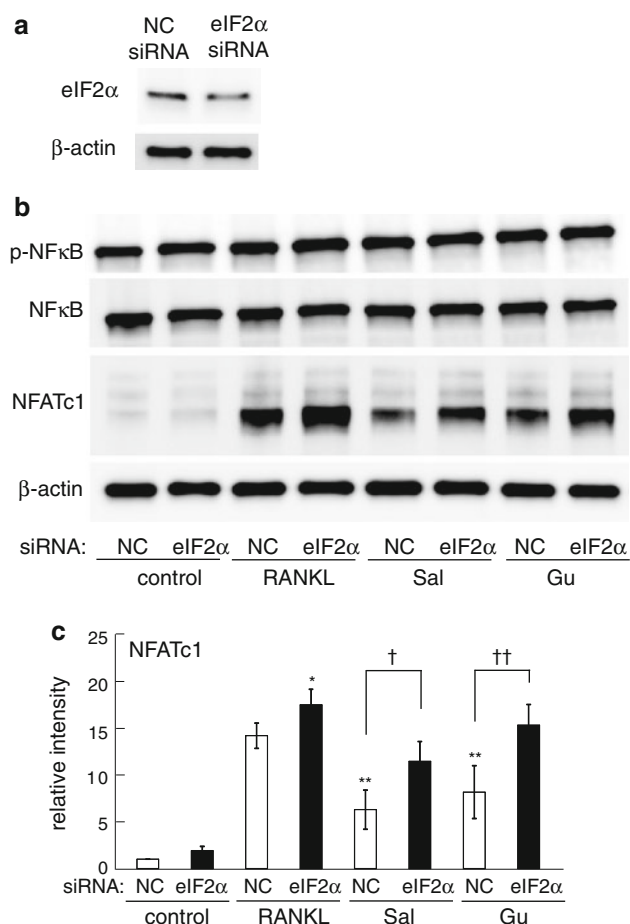


Fig. 10 Reduction in salubrinal/guanabenz driven suppression of NFATc1 expression by RNA interference specific for eIF2α. *Sal* salubrinal, *Gu* guanabenz, and *NC* non-specific control siRNA. The *single and double asterisks* indicate significant changes to the RANKL-treated NC siRNA cells with $p < 0.05$ and $p < 0.01$, respectively. The *single and double daggers* indicate significant changes to the salubrinal or guanabenz-treated NC siRNA cells with $p < 0.05$ and $p < 0.01$, respectively. **a** eIF2α level after transfecting siRNA specific to eIF2α. **b** Western blot analysis of p-NFκB and NFATc1. **c** Comparison of the expression level of NFATc1 between control siRNA and eIF2α siRNA

primary bone marrow derived cells support salubrinal's efficacy on inhibition of bone resorption. In summary, we demonstrate that elevation of p-eIF2α stimulates osteocalcin expression through upregulation of ATF4 in osteoblasts and inhibits TRAP expression via downregulation of NFATc1 in pre-osteoclasts. Silencing eIF2α with RNA interference reduced suppression of salubrinal/guanabenz-driven downregulation of NFATc1. The results in this study support the possibility of regulating bone remodeling through eIF2α-mediated signaling for combatting bone loss in osteoporosis.

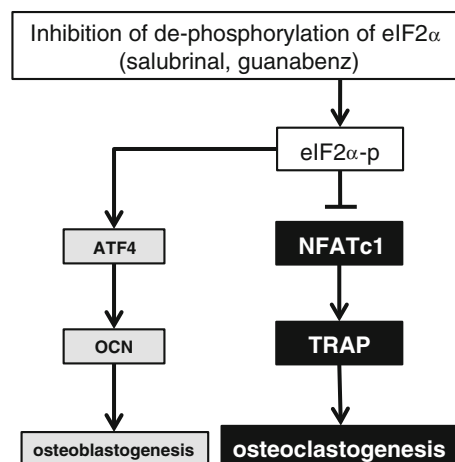


Fig. 11 Proposed mechanism of eIF2α signaling on osteoclastogenesis through NFATc1

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Conflict of interest All authors state that they have no conflicts of interest.

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